



# MicroRNAs in Normal and Malignant Lymphocytes

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**MicroRNAs in normal and malignant lymphocytes****Abstract**

MicroRNAs (miRNAs) are 20-22 nucleotide non-coding RNAs that can play important roles in developmental transitions by post-transcriptional regulation of mRNA translation and stability. We profiled miRNA expression in mouse thymocytes, mature T cells, and activated T cells, and identified distinctive patterns of miRNA expression during development, maturation, and activation of T cells. The miR-128 and miR-181 miRNA families are expressed at significantly higher levels in thymocytes. Examining the expression levels of these microRNAs in more detail, we observed that the expression pattern of these microRNA families distinguishes cells committed to lymphoid lineages from cells committed to myeloid lineages during normal mouse hematopoiesis. Extending this work to human malignancies, we determine that high miR-128 expression distinguishes lymphoid precursor derived malignancies from myeloid precursor derived malignancies.

Little information is available regarding miRNA expression early after CD8 T cell activation. We demonstrate dynamic miRNA expression during early CD8 T cell activation, including the repression of miR-150, miR-181a, miR-26, miR-29 and miR-30, and the induction of miR-155, miR-31, miR-146, and the miR-17-92 cluster. We show that miR-31 is induced by calcium/Calcineurin signaling during acute CD8 T cell activation, and demonstrate elevated miR-31 expression in regulatory and memory T cell populations. We identify miR-31 targets in primary CD8 T cells and propose a model where miR-31 induction primes CD8 T cells for activation by promoting T cell survival, activation, and proliferation.

Activation induced miRNA expression patterns are also found in some human malignancies. Chronic lymphocytic leukemia is typically thought to be a disease of resting lymphocytes. However, we demonstrate an activated B cell miRNA expression signature in CLL.

Similarities in miRNA expression between activated B cells and CLL cells include high expression of miR-34a, miR-155, and miR-342-3p and low expression of miR-103, miR-181a and miR-181b. Additionally, we show that decreased levels of miR-29c and miR-223 in CLL are negative prognostic markers associated with shorter time to first therapy. These data indicate an activated B cell status for CLL cells and suggest that the expression level of individual miRNAs may predict clinical course in CLL.

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## **Attributions**

Samples of mouse HSC, CLP, CMP, GMP, and MEP progenitors profiled in Figure 2.3 were obtained from the lab of Dr. Koichi Akashi.

Patient samples from CML, AML, and ALL, together with normal donor CD34+ cells (Figure 2.4) were obtained from Dr. Cathy Wu.

Data in chapter 3 was produced in close collaboration with Dr. Shuqiang Li, who performed the Luminex and RT-PCR profiling discussed in Chapter 3, as well as Dr. Carl Novina. Dr. Lillian Werner performed the analysis of miRNA expression, clinical markers, and time to first treatment presented in Table 3.2 and Figure 3.4.

Computational prediction of NFAT binding sites and CHIP presented in Figure 4.3 was performed by Dr. Dimitrios Iliopoulos.

## Abbreviations

Ago	Argonaute
AICD	activation induced cell death
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
BCR	B cell receptor
bp	base pair
CHIP	chromatin immuno-precipitation
CLL	chronic lymphocytic leukemia
CLP	common lymphocyte progenitor
CM	central memory
CML	chronic myeloid leukemia
CMP	common myeloid progenitor
CyA	cyclosporine A
DC	dendritic cell
DN	double negative
DP	double positive
EM	effector memory
ES	embryonic stem cell
FISH	fluorescence in situ hybridization
GC	germinal center
GMP	granulocyte monocyte progenitors
GSEA	gene set enrichment analysis
HSC	hematopoietic stem cells
IFN	interferon
IgVH	immunoglobulin heavy chain variable region
LPS	lipopolysaccharide
MEP	megakaryocyte erythrocyte progenitors
MHC	major histocompatibility complex
miRNA	microRNA
NFAT	nuclear factor of activated T cells
NK	natural killer
nt	nucleotide
p-bodies	processing bodies
pre-miRNA	precursor miRNA transcript
pri-miRNA	primary miRNA transcript
PP6	protein phosphatase 6
RACE	rapid amplification of cDNA ends
RISC	RNA induced silencing complex
RT-PCR	real-time polymerase chain reaction
SP	single positive
TCR	T cell receptor
TLR	Toll like receptor
TNF- $\alpha$	Tumor necrosis factor alpha
Treg	regulatory T cell



## **Chapter 1**

### **General introduction**

MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally regulate gene expression by annealing to complementary regions within mRNAs. The first miRNA (lin-4) was discovered by classical genetic screening in *C. elegans* [1, 2]. More recent research has found that miRNAs comprise a large and conserved class of regulatory RNAs in phyla as diverse as plants, arthropods, and chordates [3-5]. miRNAs have been shown to play critical roles in the hematopoietic system, where miRNA activity is important in shaping lineage choice, supporting differentiation, and modulating the immune response.

### ***miRNA transcription and post-transcriptional processing***

More than 700 miRNAs have been identified in the mouse genome [6]. The majority of these miRNA genes resemble protein-coding genes in that they are transcribed by RNA polymerase II as capped, polyadenylated, and sometimes spliced 100-1,000 nucleotide (nt)-long primary miRNA transcripts (pri-miRNAs) containing one or several miRNA stem loops [7, 8]. One major distinction between protein-coding genes and miRNAs is that the mature miRNA can be located within either an exon or an intron of the transcript. This provides the potential for a single transcript to code for both a protein and a miRNA, and the majority of mammalian miRNAs are found within introns of protein-coding genes [9, 10]. These miRNAs are thought to be co-transcribed with their host gene, although a few intronic miRNAs such as miR-21 can be transcribed from an independent promoter [11, 12]. Additionally, a rare subset of miRNAs is transcribed by RNA polymerase III [10] .

The pri-miRNA transcript is processed in the nucleus by the Microprocessor complex minimally composed of the RNase III enzyme Drosha and the RNA binding protein DGCR8. This complex binds to and cleaves the miRNA hairpin, producing a ~70 nt precursor miRNA (pre-miRNA) [13]. The pre-miRNA is exported through the nuclear membrane by Exportin-5 and Ran-GTP [14, 15]. Some intronic miRNAs, known as mirtrons, bypass Drosha/DGCR8

processing and are produced directly as pre-miRNAs by splicing and debranching enzymes [16]. Once in the cytoplasm, the pre-miRNA loop is removed by another RNase III enzyme Dicer that cuts 21-23 nt from the base of the pre-miRNA, leaving a 21-23 nt staggered miRNA duplex. One strand of this duplex (the miRNA strand) is then incorporated into the Argonaute (Ago) protein which forms the core of the RNA-induced silencing complex (RISC), while the complementary strand (the miRNA\* strand) is degraded. While it is possible for either strand of the duplex to be incorporated into RISC, most miRNAs display a significant strand incorporation bias, with the miRNA strand predominating in RISC and its complementary miRNA\* strand found at significantly lower levels in RISC [17].

### ***miRNA dependent gene silencing***

The mature miRNA mediates the sequence-specific binding of RISC to mRNAs complementary to the miRNA. Full complementarity leads to mRNA cleavage. However, this has been shown to occur only for one animal miRNA-mRNA pair [18]. Instead, almost all mammalian miRNA-mRNA pairings involve partial complementarity between the miRNA and sites in the 3' untranslated region (3'UTR) of its mRNA targets. Most important to this mode of binding is the 8 nt “seed” region at the 5' end of the miRNA, with 3' complementarity being of secondary importance [19]. Because of the importance of the seed region to binding, miRNAs are grouped into families with shared seed sequences, and members of these families are predicted to regulate overlapping sets of genes. Computational methods predict that more than half of all protein-coding genes may be regulated by miRNAs, suggesting that miRNAs play an important and poorly understood role in gene regulation [20, 21].

miRNA-mediated RISC binding represses target mRNA expression through at least two mechanisms: translational inhibition and/or mRNA destabilization. Translational inhibition by miRNAs appears to act at the level of translational initiation, either by RISC competing with the

EIF4E initiation factor for the m<sup>7</sup>G cap binding, or by inhibiting joining of the 60S ribosomal subunit to the 40S ribosomal subunit [22, 23]. Transcript destabilization may involve selective recruitment of deadenylases by the GW182/TNRC6 component of RISC, followed by decapping and mRNA degradation in processing bodies (P-bodies) [24, 25].

### ***miRNAs in hematopoietic development***

The first miRNA was discovered in *C. Elegans* among the set of heterochronic genes, which drive cells to differentiate into the appropriate cell types for each developmental stage [1]. miRNA function is also essential for mammalian development, as loss of the Dicer or Drosha biosynthetic enzymes results in an embryonic lethal phenotype [26, 27]. Knockout embryos fail to form a body plan during gastrulation, and it has been impossible to isolate viable Dicer knockout embryonic stem cell (ES) lines [28].

Hematopoiesis is a well-studied model of mammalian development. A small number of self-renewing multipotent hematopoietic stem cells (HSC) produce multiple mature cells to carry out the many functions of the hematopoietic system. Tight regulation of gene expression is essential to hematopoiesis, with well-known roles for the coordinated, stage specific expression of a network of transcription factors [29]. However, recent research has found that post transcriptional regulation by miRNAs also plays an important role in lineage specification and hematopoietic development. The essential role of miRNAs in the hematopoietic system has been demonstrated with conditional knockouts of Dicer in immune lineages. Dicer knockout in B cell progenitors results in a nearly complete block in development in the pro-B to pre-B transition, which could be partially reversed by the ablation of a single miRNA target [30]. Dicer knockout early in the T cell lineage does not lead to a developmental block, although T cell development is significantly impaired [31]. Peripheral T cells are more strongly affected by Dicer deletion, with defects in maturation, homeostatic proliferation, and antigenic response [32].

Specific miRNAs have been identified which function during hematopoiesis to support HSC, or promote development of specific lineages, such as miR-125b and miR-451. miR-125b is highly expressed in HSC, and is expressed at progressively lower levels in more differentiated subsets. Ectopic expression of miR-125b increased HSC engraftment and output [33, 34]. miR-125b represses pro-apoptotic proteins, such as Bmf and Klf13, and it is likely that repression of these proteins is partially responsible for the increase in HSC survival during engraftment and subsequent output. In contrast, miR-451 acts to promote terminal differentiation of the erythroblast hematopoietic lineage, thus regulating red blood cell production. miR-451 expression is strongly induced in erythroid tissues by the transcription factor GATA-1, and knockout of miR-451 in mice impairs complete maturation of erythrocytes, leading to erythroid hyperplasia, splenomegaly, and anemia [35].

Individual miRNAs have also been shown to promote or block differentiation into lymphoid lineages. Chen *et al.* published the first study linking miRNA expression to lineage differentiation [36]. Chen *et al.* observed high expression of miR-181a in bone marrow and thymus, and found that ectopic expression of miR-181a in HSC resulted in an increase in B lineage cells *in vivo*. A second miRNA that plays an important role in hematopoiesis is miR-150, which is selectively expressed in mature T cells and B cells. [37]. miR-150 targets c-Myb, an important transcription factor expressed in developing but not mature B lineage cells [38]. Ectopic expression of miR-150 in developing B cells blocks differentiation at the ProB to PreB transition, whereas miR-150 deletion leads to elevated levels of B1 B lineage cells [38].

### ***miRNAs in the immune response***

In addition to their regulation of hematopoietic development, miRNAs also play important roles during the immune response. Careful control of the immune response is required to maximize anti-pathogen activity while preventing excessive and deleterious immune reactions.

miR-146a and miR-155 are selectively expressed in activated cells of the immune system. [39-43], and studies utilizing knockout mice have revealed that miR-155 and miR-146a play essential roles in tuning the immune response to pathogens.

Research into the immunological role of miR-155 was greatly advanced by the production of miR-155 knockout mice by two independent groups, Rodriguez *et al.* and Thai *et al.* [44, 45]. Consistent with the observed miR-155 expression pattern, both groups observed normal hematopoietic development followed by serious defects in immune function. B cells from miR-155 knockout mice secreted lower levels of IgM and reduced class switched antibodies after immunization, a phenotype associated with defective germinal center formation. Thai *et al.* attribute the changes in GC formation to deficiencies in the production of the GC-promoting chemokines lymphotoxin- $\alpha$  and tumor necrosis factor by miR-155 knockout B cells. miR-155 knockout CD4 T cells displayed a pronounced bias towards secretion of Th2 type cytokines, which Rodriguez *et al.* link to the deregulation of the miR-155 target c-Maf, a Th2 specific transcription factor [46]. The defects in immune function in miR-155 mice prevented them from mounting a protective immune response to bacterial vaccinations. In addition, Rodriguez *et al.* also observed autoimmune phenotypes in the lungs of miR-155 knockout mice. More recent research has found that miR-155 is induced in regulatory T cells (Treg) by the Foxp3 transcription factor, and promoted Treg development [47]. These results show that miR-155 plays important roles in initiating an effective adaptive immune response and in preventing autoimmunity.

miR-146a is induced in myeloid cells after stimulation with Toll like receptor (TLR) ligands or pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- $\alpha$ ) [48]. miR-146a is also upregulated during the early activation of CD4 T cells, and displays biased expression in differentiated CD4 Th1 T cells [37, 49]. miR-146a knockout mice produced by Boldin *et al.* exhibit hyper-inflammatory responses to TLR stimulation characterized by

production of excessive amounts of pro-inflammatory cytokines [50]. In addition, aged miR-146a knockout mice develop an autoimmune condition involving splenomegaly, T cell activation, and autoantibody production. Release of pro-inflammatory signaling molecules including Irak1, Irak2, and Traf6 from miR-146a repression contributes to this hyper-inflammatory phenotype [51]. These results indicate that miR-146a expression in activated T cells and myeloid cells drives a negative feedback loop which attenuates the immune response and prevents excessive inflammation.

### ***miRNAs in hematopoietic malignancies***

The importance of miRNAs to the hematopoietic system has been emphasized by the links between miRNAs and hematopoietic malignancies. Many miRNAs display similar expression between cancers and their originating cell type. This miRNA fingerprint links cancers to normal cell types and provides clues to malignant ontogeny [52]. However, some miRNAs have cancer specific expression. Cancer specific miRNA expression can serve as a prognostic marker, and a subset of miRNAs have been shown to directly function as oncogenes or tumor suppressors [53]. miRNA loci are often located in genomic regions that are deleted or amplified in cancers, including those of hematopoietic origin [54]. One example was published for chronic lymphocytic leukemia (CLL), where Calin *et al.* reported that two miRNA genes, miR-15a and miR-16-1, were located at a chromosomal region frequently deleted in CLL [55]. miR-15a and miR-16 repress Bcl-2, and de-repression of this anti-apoptotic gene is linked to defective apoptosis observed in CLL [56]. The first demonstrated oncogenic miRNA was miR-155, encoded by the BIC locus. Ectopic expression of miR-155 was shown to synergize with Myc to drive erythroleukemogenesis and lymphomagenesis in chickens [57]. Expression of miR-155 alone in mouse B cells is sufficient to drive aberrant cellular proliferation and development of leukemia and lymphoma in mice [58]. miR-155 targets in hematological malignancies include

Smad5 and Ship1[59, 60]. miR-155 expression is a signature of many cancers of hematopoietic and non-hematopoietic origin, indicating the potent oncogenic potential of this miRNA (reviewed in [61]).

miRNAs clearly play an important role in regulating cellular differentiation, with many of the most definitive demonstrations of this role found in the hematopoietic system. Further studies on miRNA expression and function during hematopoiesis will serve to unravel the complicated network of gene regulation which underlies differentiation and lineage choice, as well as providing a deeper understanding of miRNA function. miRNA knockout data indicates that the adaptive T cell response is tuned and regulated by selective miRNA expression. However, most published studies have focused on the role of miRNAs in CD4 T cells, with relatively little known regarding the role of miRNAs in CD8 T cells. Investigation of miRNA expression and activity in the CD8 T cell compartment will improve our understanding of the role of miRNA regulation of the cytotoxic T cell response.

Comparison of miRNA expression in normal and malignant hematopoietic tissues can provide insight into oncogenesis. First, miRNA profiles link tumors to their cell type of origin. Second, miRNA expression patterns can be associated with prognosis, and can thus provide useful clinical information. Third, loss and gain of miRNA expression can drive oncogenesis, raising the possibility of miRNA based therapeutics.



## **Chapter 2**

**miR-128 and miR-181 family members are selectively induced in lymphoid  
lineages during hematopoiesis**

## **Abstract**

MicroRNAs (miRNAs) are 20-22 nucleotide non-coding RNAs that can play important roles in developmental transitions by post-transcriptional regulation of mRNA translation and stability. We profiled miRNA expression in mouse thymocytes, mature T cells and activated T cells and identified miRNAs selectively expressed in developing thymocytes, the majority of which derived from the miR-128 and miR-181 miRNA families. Examining the expression levels of these miRNAs in more detail, we observed that the expression pattern of these miRNA families distinguishes cells committed to the lymphoid lineages from cells committed to myeloid lineages in both normal mouse hematopoiesis and in human hematopoietic malignancies.

## Introduction:

Hematopoiesis proceeds through the production of multiple mature cell types from self-renewing multipotent hematopoietic stem cells (HSC), a process that involves the tight regulation of large networks of genes. Regulation of transcription by classical transcription factors is essential to this process [29]. However, recent research has found that post-transcriptional regulation by microRNAs (miRNAs) also plays an important role in lineage specification and hematopoietic development.

Mature hematopoietic cells are derived from the progressive restriction of cell fate in increasingly specialized progenitor cells. One of the earliest identified lineage restrictions occurs in the progenitor cells that give rise to lymphoid and myeloid cells, the two major lineages of hematopoietic cells. The common lymphocyte progenitor (CLP) can give rise to all lymphoid lineages, including T, B, and natural killer (NK) cells but has limited myeloid developmental potential [62, 63]. In contrast, the common myeloid progenitor (CMP) can give rise to all the myeloid cell types, but not to lymphoid cells [64]. Downstream of the common myeloid progenitor are two increasingly specialized myeloid lineage progenitors: the megakaryocyte/erythrocyte progenitors (MEP), which differentiate into platelets and red blood cells, and the granulocyte monocyte progenitors (GMP), which give rise to the remaining myeloid cell types including macrophages, mast cells, granulocytes, and monocytes [65]. After commitment to the lymphoid lineage, cells develop into T cells and B cells in the thymus and bone marrow through distinct stages which correspond to the progressive rearrangement of the T cell receptor (TCR) and B cell receptor (BCR) *loci* and selection of the antigen repertoire.

Strong evidence for the essential roles of miRNAs in hematopoiesis comes from conditional deletion of the key miRNA biogenesis enzyme Dicer. Loss of Dicer in HSCs results in a loss of the long-term repopulating stem cell pool *in vivo* [66]. Loss of Dicer in B or T cell

precursors results in significant developmental blocks and aberrant differentiation of mature cells [30, 67]. In addition, specific miRNAs have been shown to regulate the development of individual hematopoietic lineages (reviewed in [68]). There is substantial overlap between factors which regulate normal hematopoiesis and those which are dysregulated in hematological cancers. Thus, tracking expression of miRNAs during normal and malignant hematopoiesis may yield important insights into the shared and divergent roles that miRNA regulation plays in hematopoiesis and leukemogenesis.

In the current investigation, we produced a detailed profile of miRNAs expressed during T cell development. We identified two families of miRNAs, miR-128 and miR-181, which are selectively expressed in developing T cells. Examining the expression of these two families in cells from other hematopoietic lineages, we found that the expression of these two miRNAs distinguish lymphoid lineages from myeloid lineage cells in both normal murine hematopoiesis and lymphoid malignancies.

## **Materials and Methods**

### ***Mice***

Male c57bl/6 mice between 5-10 weeks of age were purchased from Taconic Farms (Hudson, NY, USA).

### ***Patient samples and cell preparation***

Samples were obtained from patients and normal donors enrolled on clinical research protocols at the Dana-Farber Cancer Institute approved by the DFCI Human Subjects Protection Committee. Peripheral blood mononuclear cells were isolated by Ficoll/Hypaque density gradient centrifugation, cryopreserved with 10% DMSO, and stored in vapor-phase liquid nitrogen until the time of analysis. CD34<sup>+</sup> cells from normal donor bone marrow aspirate were isolated by means of a fluorescence-activated cell sorter.

### ***Total RNA preparation***

Total RNA was extracted by the Trizol method (Invitrogen, Carlsbad, CA, USA). Dried RNA pellets were re-suspended in appropriate volumes of nuclease-free water. RNA was quantitated by O.D.<sub>260/280</sub> using a spectrophotometre DU640 (Beckman).

### ***miRNA expression profiling***

We profiled 279 miRNAs by the Luminex method as described elsewhere [52]. Profiling data was normalized by generating a mean array for the expression of each miRNA in all samples. Next, a linear correction for each sample to the mock array was performed. Hierarchical clustering was performed using GenePattern software from the Broad Institute [69].

### ***miRNA-specific RT-PCR assay***

miRNA specific cDNA synthesis from total RNA was carried out using MicroRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). miRNA-specific RT-PCRs were performed using Taqman miRNA assays according to the manufacturer's protocol (Applied Biosystems, Carlsbad, CA, USA). miRNA expression was calculated using a standard curve derived from a matched synthetic single stranded miRNA.

### ***Fluorescence activated cell sorting for miRNA profiling:***

Samples of mouse HSC, CMP, GMP, and MEP were obtained from the laboratory of Dr. Akashi and isolated as previously described [62, 64, 65]

For isolation of thymocytes, thymocytes were stained with  $\alpha$ -CD117,  $\alpha$ -CD44,  $\alpha$ -CD25,  $\alpha$ -CD8,  $\alpha$ -CD4, and  $\alpha$ -CD24 antibodies. Cells were isolated as follows: early CD8 SP (CD8<sup>+</sup> CD4<sup>-</sup> CD24<sup>high</sup>); late CD8 SP (CD8<sup>+</sup> CD4<sup>-</sup> CD24<sup>low</sup>); DP (CD4<sup>+</sup> CD8<sup>+</sup>); DN (CD4<sup>-</sup> CD8<sup>-</sup>). DN cells were further subdivided by expression of CD117, CD44, and CD25 as follows: DN1 (CD117<sup>high</sup> CD44<sup>high</sup> CD25<sup>-</sup>); DN2 (CD117<sup>high</sup> CD44<sup>high</sup> CD25<sup>+</sup>); DN3 (CD117<sup>med</sup> CD44<sup>med</sup> CD25<sup>+</sup>) ; DN4 (CD117<sup>low</sup> CD44<sup>low</sup> CD25<sup>-</sup>). For isolation of mouse B cell progenitors, red blood cells were removed from mouse bone marrow with red blood cell lysing buffer (Sigma, St. Louis, MO, USA). Cells were stained with a panel of non-B lineage markers ( $\alpha$ -CD3,  $\alpha$ -GR1,  $\alpha$ -TER-119,  $\alpha$ -CD11B and  $\alpha$ -DX5), and  $\alpha$ -CD117,  $\alpha$ -CD127,  $\alpha$ -CD19 and  $\alpha$ -B220. Cells were isolated as follows: CLP (Lin<sup>-</sup> CD19<sup>-</sup> B220<sup>-</sup> CD117<sup>+</sup> CD127<sup>+</sup>); ProB (Lin<sup>-</sup> CD19<sup>-</sup> B220<sup>+</sup> CD117<sup>+</sup> CD127<sup>+</sup>); PreB1 (Lin<sup>-</sup> CD19<sup>+</sup> B220<sup>+</sup> CD117<sup>+</sup> CD127<sup>+</sup>); PreB2 (Lin<sup>-</sup> CD19<sup>+</sup> B220<sup>+</sup> CD117<sup>-</sup> CD127<sup>+</sup>). For isolation of mature T cells and B cells, red blood cells were removed from mouse bone marrow with red blood cell lysing buffer, and cells stained with  $\alpha$ -CD19,  $\alpha$ -B220, and  $\alpha$ -CD3. B cells were isolated as CD19<sup>+</sup> B220<sup>+</sup>, and T cells as CD3<sup>+</sup>. All antibodies were obtained from eBioscience (San Diego, CA, USA)

### ***Primary Cell Culture and Activation***

CD8 T cells were isolated from 5-10 week old c57Bl/6 mice by negative selection using EasySep beads (Stemcell Technologies, Vancouver, BC, Canada.). T cells were cultured in RPMI-1640 Medium supplemented with 10% fetal calf serum, 50 units/ml Penicillin-Streptomycin, 10 mM HEPES, 2 mM L-glutamine, and 55  $\mu$ M 2-mercaptoethanol (GIBCO, Carlsbad, CA, USA). Cells were cultured at 37°C with 5% CO<sub>2</sub>. T cells were activated with 10  $\mu$ g/ml plate bound  $\alpha$ -CD3 and 2  $\mu$ g/ml soluble  $\alpha$ -CD28 antibodies (eBioscience, San Diego, CA, USA ), and RNA was isolated from cells 24 hours and 96 hours after activation.

BM derived dendritic cells were generated as described [70]. For dendritic cell activation, cells were cultured in media containing lipopolysaccharide (LPS) (10 ng/ml Alexis, San Diego, CA, USA) for 24 hours prior to miRNA profiling.

## Results and Discussion

### *miRNA profiling during T cell ontogeny*

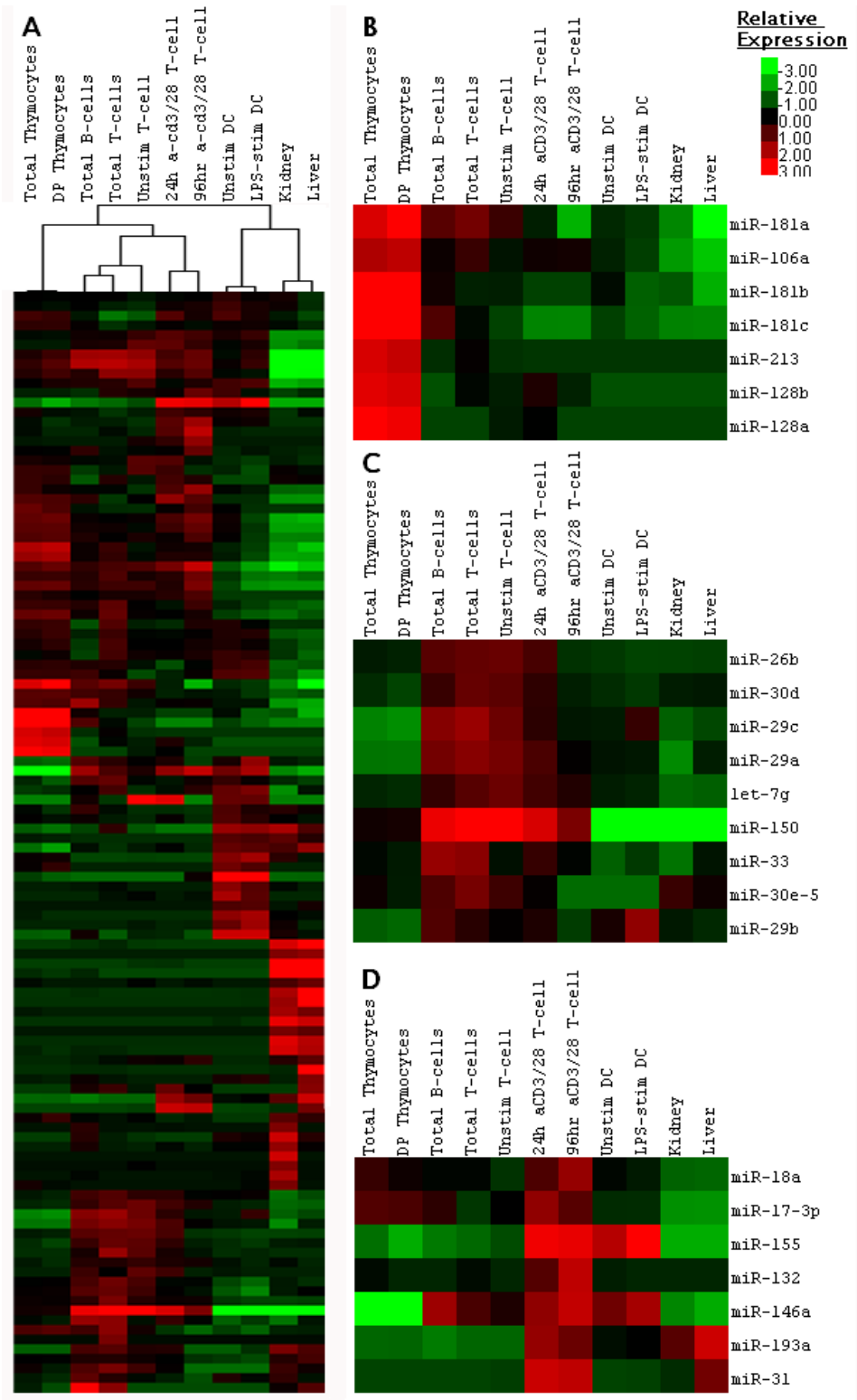
To identify miRNAs with developmental stage-specific expression during T cell ontogeny, we used a Luminex bead-based system to profile miRNA expression. Solution-phase Luminex detection can accurately distinguish between miRNAs with only a single mismatch, and offers linear detection over a hundred-fold range of expression [52]. We obtained miRNA profiles from thymocytes, mature T and B cells, activated CD8 T cells, bone marrow-derived dendritic cells (DC), and control kidney and liver tissue perfused to remove contaminating lymphocytes. Unsupervised hierarchical clustering of miRNA profiles classified samples from solid tissues, DCs, and B or T lineage cells into 3 separate groups (Figure 2.1A). Within the T lineage, distinct clusters of miRNAs are expressed in a developmental stage-specific manner. First, miRNAs which are selectively expressed in developing thymocytes in comparison to all other tissues tested (Figure 2.1B). Second, miRNAs which are selectively expressed after maturation of T and B cells (Figure 2.1C). Third, miRNAs which are induced by T cell activation, some of which are also expressed at high levels in bone marrow-derived DC or solid tissues (Figure 2.1D). Our miRNA profiles thus distinguish both lineage and T cell developmental stage.

Interestingly, the majority of miRNAs selectively expressed in thymocytes come from just two families, miR-181 (a, b, and c) and miR-128. miR-181a, b, and c family members are present in 3 separate loci. miR-213, which is also present in this cluster, is the mir\* partner for miR-181-a-1. miR-128 is present in the mouse genome at two loci. These two loci were initially thought to produce miR-128a and miR-128b, miRNAs with a 1 nt difference at the 3'-end. However, deep sequence data indicates that the most commonly produced mature miRNAs from these two loci are identical, so they have been renamed miR-128-1 and -2 [71]. The coordinated induction of multiple family members from separate loci suggests that expression of miR-128 and



**Figure 2.1: Luminex profiles of miRNA expression in T lineage and control tissues. A:** Pairwise complete linkage hierarchical clustering of all miRNAs expressed at >3 standard deviations over background in at least one sample. Samples include total thymocytes, DP thymocytes, mature T cells and B cells, 24 hour and 96 hour  $\alpha$ -CD3/CD28 activated CD8 T cells, bone marrow-derived DC, lipopolysaccharide (LPS) activated DCs, and control kidney and liver tissue perfused to remove contaminating lymphocytes. Data represents expression relative to the mean value for each row. Groups of miRs are differentially expressed in developing (**B**), mature (**C**), and activated T cells (**D**).

Figure 2.1 (Continued)



miR-181 family members may be a distinctive feature of T cell development.

***Real-time PCR validates miR-128 and miR-181 expression during T and B cell development***

To independently validate the expression of miR-128 and miR-181 family members, we examined the expression of these miRNAs during T cell development in more detail by real time PCR (RT-PCR) [72]. RT-PCR can operate efficiently with limited biomass, allowing us to profile the expression of miR-128 and miR-181 in low-frequency populations, including individual thymocyte subsets.

Thymocyte development proceeds through multiple stages, which can be distinguished by the expression of surface markers (Figure 2.2A). Cells in the earliest stage of T cell development lack CD4 and CD8 surface expression, and are known as double negative cells (DN). DN can be further subdivided into 4 subsets by the expression of CD25, CD44, and CD117 (c-kit) (Figure 2A). Inclusion of the CD117 marker is essential for the isolation of DN1 and DN2 T-lineage cells since (a) expression of the CD44 marker is common among non-T lineage cells and (b) the majority of the T cell developmental potential present within the DN1 stage is restricted to the small population of cells which also express high levels of CD117 [73]. During the double negative stage, cells lose the ability to commit to non-T lineages (DN1-2), and rearrange the TCR- $\beta$  locus (DN2-3) [74]. Next follows the double positive stage (DP), in which cells express both CD4 and CD8, and undergo TCR- $\alpha$  rearrangement. Starting at the DP stage, thymocytes go through a process of positive selection, which enables survival of thymocytes which have successfully rearranged a TCR with the capability to bind to major histocompatibility complex (MHC) molecules, and negative selection, which eliminates thymocytes with high affinity for self antigens that could pose a risk of autoimmunity[75]. Single positive cells (SP)

express either CD4 or CD8, and continue to go through negative selection as they complete maturation in the thymus, which corresponds to a decline in CD24 expression [76, 77].

We purified thymocyte populations by FACS according to surface marker expression and tested their miRNA expression. RT-PCR results found high expression of both miR-128 and miR-181 family members, confirming our Luminex profile data. However, comparison of thymocyte subsets revealed distinct changes in miRNA expression over the course of T cell development. High expression of both miR-128 and miR-181 family members was observed at the earliest DN1 stage of T cell development. Expression dipped at the DN2 stage, recovered at DN3, and then progressively declined during subsequent thymocyte development, with the expression of miR-128, miR-181b and miR-181c lost as single positive cells matured and left the thymus. miR-181a expression was increased at the early single positive stage, and was the only signature miRNA to remain expressed in mature T cells, albeit at significantly lower levels (Figure 2.2B). These data confirm our Lumiex profiles, and reveal dynamic regulation of miRNA levels at individual stages of thymocyte development.

Despite the known importance of miRNAs to T cell development, there are relatively few publications examining miRNA expression in detailed thymocyte populations. Nielson *et al.* profiled miRNA expression during thymocyte development by cloning and sequencing short RNAs [78]. Consistent with our results, these authors observed expression of miR-128 and miR-181 family members in thymocyte subsets, with a peak in miR-128 expression at the DN4 stage, and a peak in miR-181a and b expression at the DP stage. Neilson *et al.* did not profile DN2 stage thymocytes, nor did they observe miR-128 or miR-181 family enrichment in the DN1 thymocyte stage [78]. Li *et al.* tracked miR-181a expression during thymocyte development, and reported peak expression at the DN3 stage, with lower expression in DN1 and DN2 [79]. Both of these groups defined the DN1 stage using the classical surface marker pattern of DN CD44<sup>+</sup> CD25<sup>-</sup>. However, a large fraction of this population consists of non-T lineage cells which could interfere

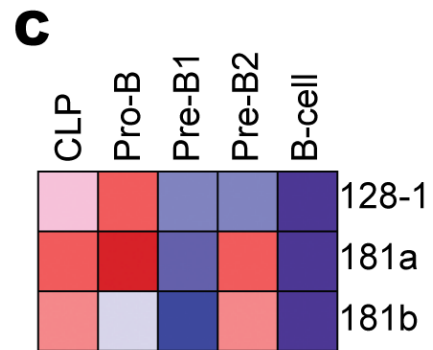
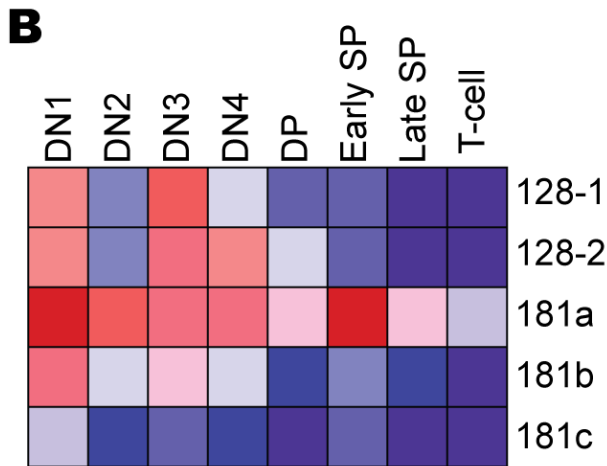
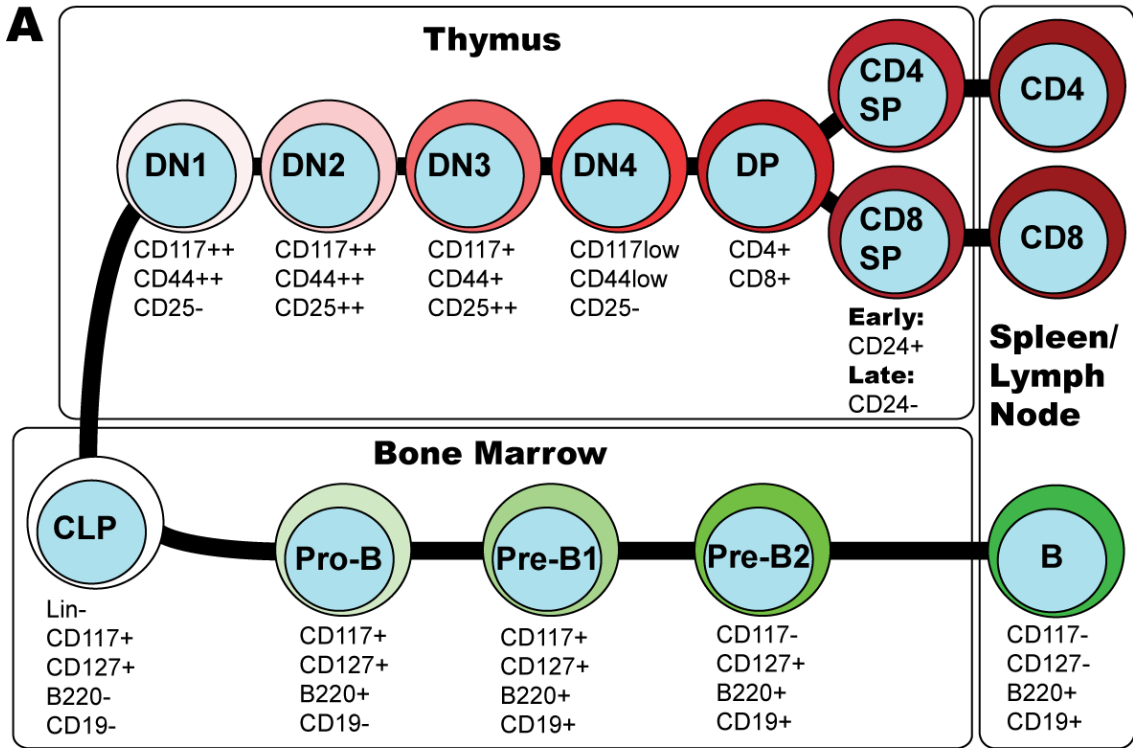
with the detection of lineage-specific miRNA expression. By carefully isolating the CD117-high T-lineage progenitors from the bulk DN1 population, we were able to detect the distinctive expression of miR-128 and miR-181 family members in the earliest definitive T cell progenitor in the thymus.

In order to determine if expression of miR-128 and miR-181 is T cell specific or a general feature of lymphoid development, we also profiled miRNA expression in the B cell lineage by RT-PCR. B cell development from the CLP in the bone marrow is highly analogous to T cell development, with subpopulations at distinct developmental stages bearing unique patterns of surface marker expression (Figure 2.2A). Pro-B cells are committed to the B lineage, Pro- and Pre-B1 cells rearrange the immunoglobulin heavy locus, and Pre-B2 cells rearrange the  $\kappa$  or  $\lambda$  light chain locus prior to the final stages of B cell maturation in the periphery [74]. We sorted CLP and developing B cell subtypes from bone marrow and tested miRNA expression by RT-PCR. Strong expression of miR-128 and miR-181a and b was found in developing B cells at the Pro-B and Pre-B2 stage. Pre-B1 stage cells expressed lower but still detectable levels. Most notably, miR-128 and miR-181a and b expression was high in bone marrow CLP cells. These data indicate that miR-128 and miR-181 family expression is a feature of both developing B and T-lineage cells, and that induction of these miRNAs occurs before commitment to the T cell or B cell lineage.

The expression of miR-181 but not miR-128 has previously been reported in B cell development. miR-181a was one of the first miRNAs identified as being differentially expressed during hematopoiesis. Chen *et al.* demonstrated that miR-181 was overexpressed in mouse bone marrow and thymus [36]. Spierings *et al.* profiled miRNA expression by deep sequencing during mouse B cell development, and confirmed high expression of miR-181 in early B cell subsets [80]. Our experiments indicate that miR-128 is a marker of B cell development, and confirm previous research regarding the expression of miR-181 during B cell development.

**Figure 2.2: miR-128 and miR-181 family members are expressed during both B and T cell development.** **A)** Stepwise developmental stages during T cell development in the thymus and B cell development in the bone marrow, culminating in mature T and B cells in the periphery. **B)** miRNA expression in T-lineage populations of double negative (DN1-4), double positive (DP), early CD24<sup>+</sup> CD8 single positive (early SP), late CD24<sup>-</sup> CD8 single positive (late SP) cells were isolated from the thymus, and mature T cells from the spleen. **C)** miRNA expression in CLP and B-lineage cells from the bone marrow and mature B cells from the spleen. MicroRNA levels were determined by real time PCR using a standard curve of synthetic RNA input to determine absolute expression.

Figure 2.2 (Continued)

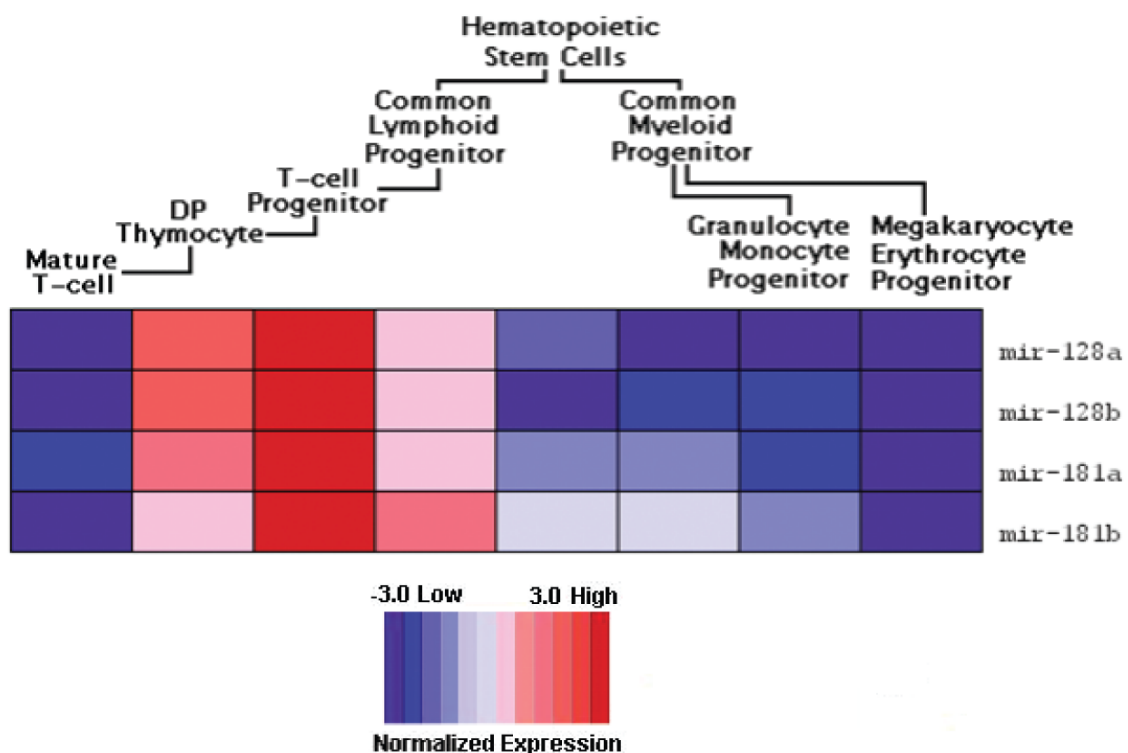


### ***miR-128 and miR-181 are markers for lymphoid commitment***

Since miR-181 and miR-128 families were expressed in the earliest T-lineage population in the thymus, the early B-lineage in the bone marrow, and in the bone marrow common lymphoid progenitor, we measured expression of these miRNA families earlier in hematopoietic development by RT-PCR to determine at what stage of development these miRNA families were induced. Both miR-128 and miR-181 family members are expressed at low but detectable levels in HSC. As commitment to the lymphoid lineage increases from HSC to CLP, and subsequently to early and late T-lineage committed cells (DN1 and DP), the expression of miR-181 and miR-128 family members also increases. In contrast, during myeloid development, as HSC differentiate to the CMP, and the CMP further differentiates into the GMP and MEP, the expression of miR-128 and miR-181 family members decreases (Figure 2.3). These data indicate that expression of miR-128 and miR-181 family members is a general feature of commitment to a lymphoid rather than myeloid cell fate.

A number of groups have profiled miRNA expression in HSC from mice and humans [33, 34]. However, there is limited information available on the expression of miRNAs in other committed hematopoietic progenitor populations. Georgantas *et al.* detected miR-128 and miR-181 expression in human HSC, and based on computational miRNA-target predictions hypothesized that expression of miR-181 and miR-128 would prevent differentiation from HSC into both lymphoid and myeloid cell types [81]. We have demonstrated a progressive increase in miR-128 and miR-181 family expression during lymphoid lineage differentiation, therefore expression of these miRNAs cannot be associated with preserving a multipotent stem cell state, though they might act to inhibit myeloid differentiation.





**Figure 2.3: Expression of miR-128 and miR-181 families distinguishes developing lymphoid and myeloid lineages.** Progenitor and mature populations were sorted from mouse spleen, thymus and bone marrow. miRNA expression in sorted progenitor and mature populations was determined by real-time PCR with a standard curve to calculate absolute expression. Populations are mature T cells, DP thymocytes, DN1 thymocyte T cell progenitors, CLP, HSC, CMP, GMP, and MEP. Row-normalized expression is shown.

### ***Functional roles of miR-128 and miR-181 in normal hematopoiesis***

The functional role of miR-128 in hematopoiesis is unknown. However, publications regarding the tumor suppressive activity of miR-128 in human tumors offer some suggestions for the role miR-128 may play in hematopoiesis. One published target of miR-128 in human glioma is the Bmi-1 stem cell renewal factor, a member of the Polycomb group of genes [82]. Bmi-1 expression plays an important role in hematopoietic stem cell renewal and in thymocyte proliferation in response to pre-TCR signaling [83, 84]. A second published target of miR-128 in acute lymphocytic leukemia is the mixed lineage leukemia gene, which is essential for the maintenance of adult HSC and myeloid/erythroid progenitors [85, 86]. These known targets, together with our expression data suggest that miR-128 may affect stem cell self-renewal, regulate the survival and proliferation of thymocytes, or repress myeloid differentiation.

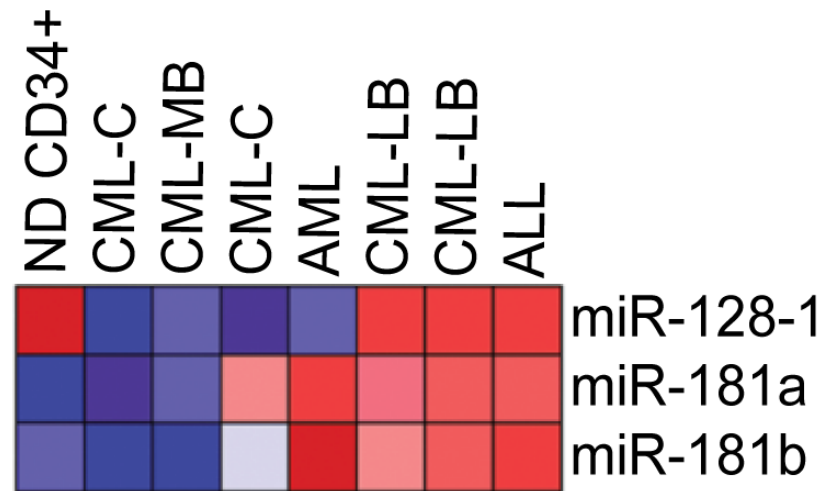
In contrast to miR-128, important regulatory roles for the miR-181 family have been identified at multiple stages of lymphoid lineage development. Expression of miR-181a in hematopoietic progenitors increases the production of lymphoid lineages appropriate to the developmental environment: ectopic expression of miR-181a in bone marrow promotes B cell development, and ectopic expression of miR-181a in early thymic progenitors promotes development to the DP stage [36, 87]. The mechanism by which miR-181a affects early development into lymphoid lineages remains to be established. miR-181a also plays an important role in shaping the developing T cell repertoire and in regulating the activation of mature T cells. In this role, miR-181a acts to decrease the minimum threshold for TCR signaling by repressing a group of inhibitory phosphatases which target pathways downstream of TCR triggering [79, 88]. High expression of miR-181a during late thymocyte development partially explains the decreased threshold for TCR triggering during positive and negative selection. Other groups have identified

additional miR-181a targets in thymocytes, including Bcl-2, CD69, and TCR $\alpha$ , which may also play a role in modulating thymocyte selection [78].

### ***miR-128 and miR-181 expression in hematopoietic malignancies***

Many leukemias exhibit the clonal expansion of cells which exhibit maturation defects at specific points in hematopoietic differentiation. Acute myeloid leukemias (AML) are myeloid precursors blocked at a variety of stages in development, whereas acute lymphoblastic leukemias (ALL) occur in immature B or T cell precursors [89, 90]. In contrast, chronic myeloid leukemia (CML) is a HSC disorder driven by a chromosomal fusion which produces the *bcr-Abl* oncogene [91, 92]. CML disease progression is complex: the initial chronic phase of disease is characterized by the production of abnormal levels of normal mature non-lymphoid cells, and is followed by a late stage of disease known as the blast crisis, with some patients experiencing an intervening accelerated phase with increasingly abnormal leukocyte counts [93]. The blast crisis phase is characterized by the rapid expansion of a population of immature myeloid or lymphoid cells, with clinical and hematological features similar to acute leukemia [94]. Since these malignancies are shaped by aberrant differentiation into lymphoid or myeloid pathways, we hypothesized that the miRNA expression patterns we observed in normal murine hematopoiesis might be conserved in human hematopoietic malignancies.

We tested miRNA expression in RNA isolated from patients with AML, ALL, chronic CML, myeloid and lymphoid blast phase CML, and from normal donor CD34<sup>+</sup> cells (Figure 2.4). miR-181a and b family members were expressed in both the lymphoid tumors and in some CML and AML patients. This is consistent with previous reports of elevated miR-181 expression in subsets of AML and CML. In AML, miR-181 expression is associated with less mature leukemic subtypes [95]. In CML, miR-181 expression is high during blast crisis but variable in samples from earlier disease stages [96]. miR-128 was expressed only in lymphoid tumors and normal



**Figure 2.4:** Expression of miR-181 and miR-128 distinguishes lymphoid and myeloid lineage human tumors. MicroRNA expression in sorted normal donor CD34+ bone marrow (ND CD34+) and patient tumor samples from: chronic phase CML [CML-C], myeloid blast phase CML [CML-MB], lymphoid blast phase CML [CML-LB], ALL, and AML was determined by real-time PCR. Row-normalized expression is shown.

donor CD34<sup>+</sup> cells. These data corroborate results from other groups who found that miR-128 was the most significantly over-expressed miRNA in ALL compared to AML [97]. In addition, our results indicate that expression of miR-128 is also a distinctive marker of aberrant differentiation into the lymphoid lineage in CML blast crisis.

## ***Conclusions***

We have demonstrated that developing lymphoid lineage cells express a distinct miRNA signature of miR-128 and miR-181 family members during murine hematopoiesis. These miRNAs are induced progressively as cells differentiate towards lymphoid cell fates, and expression is maintained through T cell development in the thymus and B cell development in the bone marrow. The connection between miRNA expression in the CLP and in the earliest T-lineage cells in the thymus is a unique aspect of our signature. The expression of miR-128 is conserved as a signal of lymphoid lineage in human hematopoietic malignancies.

The function of miR-181a in regulating TCR sensitivity late in T cell development has been well described. However, the mechanism by which miR-181 family members affect early T and B cell development remains to be established, and almost nothing is known about the function of miR-128. Further investigation into the role of miR-128 and the miR-181 family during normal and malignant hematopoiesis is required to understand what role miRNAs play in the intricate network of genes that regulate hematopoiesis.

## **Chapter 3**

### **MicroRNA expression profiling identifies activated B cell status in chronic lymphocytic leukemia cells**

This chapter is adapted from a published work [98].

## Abstract

Chronic lymphocytic leukemia (CLL) is thought to be a disease of resting lymphocytes. However, recent data suggest that CLL cells may more closely resemble activated B cells. Using miRNA expression profiling of highly-enriched CLL cells from 38 patients, 9 untransformed B cells from normal donors before acute CpG activation, and 5 matched B cells after acute CpG activation, we demonstrate an activated B cell status for CLL. Gene set enrichment analysis (GSEA) identified statistically-significant similarities in miRNA expression between activated B cells and CLL cells including upregulation of miR-34a, miR-155, and miR-342-3p and downregulation of miR-103, miR-181a and miR-181b. Additionally, decreased levels of two CLL signature miRNAs, miR-29c and miR-223, are associated with ZAP70<sup>+</sup> and IgV<sub>H</sub> unmutated status and with shorter time to first therapy. These data indicate an activated B cell status for CLL cells and suggest that the direction of change of individual miRNAs may predict clinical course in CLL.

## Introduction

CLL is the most common form of adult leukemia in the western world, accounting for approximately 30% of all leukemias in Caucasians. Contrary to its earlier description as a relatively homogeneous disease, CLL has recently been viewed as a heterogeneous disease with variable clinical course that correlates with several biologic markers of prognosis [99]. The most clinically significant prognostic markers are cytogenetics, as determined by fluorescence *in situ* hybridization (FISH), and immunoglobulin heavy chain variable region (IgV<sub>H</sub>) mutation, followed by ZAP70 status. Deletion of 11q or 17p, high expression of ZAP70 or CD38, and relative absence of V region somatic hypermutation are markers of more aggressive CLL disease.

CLL is characterized as a disease of mature B cells. CLL cells typically express an anergic B cell receptor (BCR) and demonstrate dysregulated apoptotic programs. mRNA expression profiling has been used to classify CLL [100-103]. Though it is not generally considered a disease of activated B cells, mRNA expression profiling characterized CLL cells as similar to activated B cells in one study [100] and similar to memory B cells in another [101]. In normal B cells, the nuclear translocation of NF- $\kappa$ B is associated with B cell activation. Constitutive nuclear localization of nuclear factor of activated T cells (NFAT) and NF- $\kappa$ B2/p52 characterizes CLL cells [104], suggesting an activated B cell state. Moreover, CLL cells demonstrate higher NF- $\kappa$ B DNA binding activity than untransformed B cells, the RelA subunit of NF- $\kappa$ B has been shown to be associated with clinical disease progression, and RelA binding activity is inversely correlated with apoptosis in CLL cells [105]. Recently, CLL cells were shown to express activated cell surface markers and intracellular phenotypes [106].

CLL has also been classified by miRNA expression profiling [107-112]. These profiles found correlations between miRNA expression and known clinically significant prognostic markers, and raised the potential for miRNAs to serve as independent prognostic markers. In



addition to their utility as markers, downregulation of several miRNAs has been linked to the apoptotic defect observed in CLL. Downregulated miR-15a and miR-16 fail to repress Bcl-2 [113] and downregulated miR-29 fails to repress Mcl-1 [114]. However, it is important to note that there is considerable variation in the published miRNA expression profiles for CLL, indicating a need for additional research in this area.

Using miRNA expression profiling, we identified a miRNA signature in untransformed B cells induced soon after activation. This activated B cell miRNA signature is also present in CLL cells indicating an activated B cell phenotype for CLL. Our data imply that individual miRNAs involved in B cell activation may participate in the B cell transformation process and could be targets for therapeutic gene silencing in CLL.

## **Materials and Methods**

### ***Control B cell donor and CLL cell patient characteristics***

Cells from 38 CLL patients were assessed in this study. All patients were enrolled on a tissue banking protocol, #99-224, prior to sample collection. This tissue banking protocol was approved by the Dana-Farber Cancer Institute (DFCI) Institutional Review Board, and informed consent was obtained from all CLL patients prior to sample collection. These patients had white blood cell counts (WBC) between  $15.7 \times 10^3$  and  $265.6 \times 10^3$  cells per microliter of blood. Three of the CLL patients were treated, while the other 35 patients were untreated. Control blood samples were from healthy donors. ZAP-70 was assessed by flow cytometry, and the expressed IgV<sub>H</sub> was determined by reverse transcription polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay as previously described [115]. ZAP-70 expression (positive defined as >20%) and IgV<sub>H</sub> homology (unmutated defined as greater than or equal to 98% homology to the closest germline match) were determined by the CLL Research Consortium tissue core. Full patient clinical parameters are published in Li *et al* [98].

### ***Control B cell and CLL cell purification***

10 mL of heparinized peripheral blood was subjected to Ficoll-Paque™ PLUS (GE Healthcare, Pittsburgh, PA, USA) density centrifugation. Control B and CLL cells were purified from peripheral blood mononuclear cells (PBMCs) by positive selection with CD19+ selection beads as suggested by the manufacturer (StemCell Technologies Inc, Vancouver, Canada), and then immediately used for RNA isolation or activation treatment.

### ***Control B cell culture and activation***

B cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (GIBCO, Carlsbad, CA, USA) supplemented with 10% human AB serum (Mediatech, Inc, Herndon, VA,

USA), 1% Penicillin-Streptomycin (GIBCO, Auckland, NZ, USA), 1% HEPES (Mediatech, Inc, Herndon, VA, USA), 1% L-glutamine (Mediatech, Inc, Manassas, VA, USA), 50 µg/mL human transferrin (Sigma, St. Louis, MO, USA) and 5 µg/mL insulin (Sigma, St. Louis, MO, USA). The cells were kept in a 37°C incubator with 5% CO<sub>2</sub>.

For TLR-mediated activation,  $5 \times 10^6$  control B cells were plated in 5 mL of medium containing lipopolysaccharide (LPS) (0.5 ng/µL, Alexis, San Diego, CA, USA), CpG (1 µM, Hycult Biotech, Canton, MA, USA, the sequence of the CpG is 5' tcgtcgtttgtcgtttgtcgtt 3'), or polyI:C (5 ng/µL, Invivogen, San Diego, CA, USA) at 37°C for 24 hours or 48 hours. For CD40 activation, NIH-3T3 CD40L or NIH-3T3 feeder cells were cultured to confluency and then irradiated for 9K by Cs-irradiator. Irradiated feeder cells were plated in 6-well plates at  $2 \times 10^5$  cells/mL, incubated overnight at 37°C, and washed twice gently with PBS before adding the B cell medium. Purified control B cells were co-cultured with the feeder cells at 37°C for 24 hours or 48 hours. For BCR activation, purified control B cells were adjusted to  $1 \times 10^7$  cells in 0.1M phosphate buffered saline (pH7.2). Goat anti IgM F(ab')<sub>2</sub> (Southern Biotechnology Associates, Birmingham, AL, USA) at 10 µg/mL was added to untransformed B cells for 20 minutes on ice. These cells were transferred to human B cell medium and cultured for 24 hours or 48 hours at 37°C.

For experiments involving inhibitors for the c-jun N-termina kinase (JNK) or mitogen-activated protein kinase/ERK kinase (MEK), cells were co-cultured with the JNK inhibitor SP600125 (50 µM, A. G. Scientific, Inc., San Diego, CA, USA) or MEK inhibitor PD98059 (50 µM Sigma, St. Louis, MO, USA) for 24 hours before the isolation of RNA for RT-PCR assays.

### ***Total RNA preparation***

Total RNA from control B or CLL cells was extracted by the Trizol method (Invitrogen, Carlsbad, CA, USA). Dried RNA pellets were re-suspended in appropriate volumes of DEPC/ddH<sub>2</sub>O. RNA was quantitated by OD<sub>260/280</sub> using a DU640 spectrophotometer (Beckman).

### ***MiRNA expression profiling***

We first profiled 435 miRNAs from 22 CLL samples and 1 control B sample by the Luminex method using sequential adapter ligation and gel extraction as described [52]. To expand our sample size, we then chose 43 miRNAs which were predicted to be differentially expressed in CLL from our previous data, 4 miRNAs from published data, and 3 control miRNAs for large scale profiling with the FlexmiR miRNA labeling kit (Luminex, Austin, TX, USA, as described [116]). In this method, 2.5 µg of total RNA was labeled with biotin, and then it was hybridized with locked nucleic acid (LNA)-modified capture probe coupled with beads. After washing away the unbound RNA samples, streptavidin-phycoerythrin (SAPE) reporter molecules were added to the reaction and the expression of miRNAs was analyzed on Luminex analyzer.

### ***Data analysis for miRNA profiling***

A mock array was generated by averaging the expression of each miRNA in all samples. Next, a linear correction for each sample to the mock array was performed. The data were analyzed using GenePattern software from the Broad Institute. miR-146b-5p was deleted from the analysis due to abnormally high background bead signals.

### ***Gene set enrichment analysis***

A B cell miRNA activation signature was defined as as miRNAs that were significantly altered with a t-test p-value of <0.05 after correction for multiple comparisons in control versus *in vitro* activated B cells. We performed independent GSEA analysis for upregulated and

downregulated miRNA sets to test for enrichment of activation signature miRNAs in control B cells versus CLL profiles as described in [117].

### ***MiRNA-specific RT-PCR assay***

Total RNA was treated with DNase I (Ambion, Austin, TX, USA), and cDNA synthesis was carried out using miScript Reverse Transcriptase kit (Qiagen, Hilden, Germany). miRNA specific RT-PCRs were performed using the miScript primer assay according to the manufacturer's protocol (Qiagen, Hilden, Germany). The relative expression of specific miRNAs was calculated by the  $\Delta\Delta C_t$  method. A randomly chosen subset of the CLL cell RNAs samples used for Luminex bead-based miRNA expression profiling were used for validation by RT-PCR-based miRNA expression profiling. Full RT-PCR expression data is published in Li *et al* [98].

### ***Statistical methods***

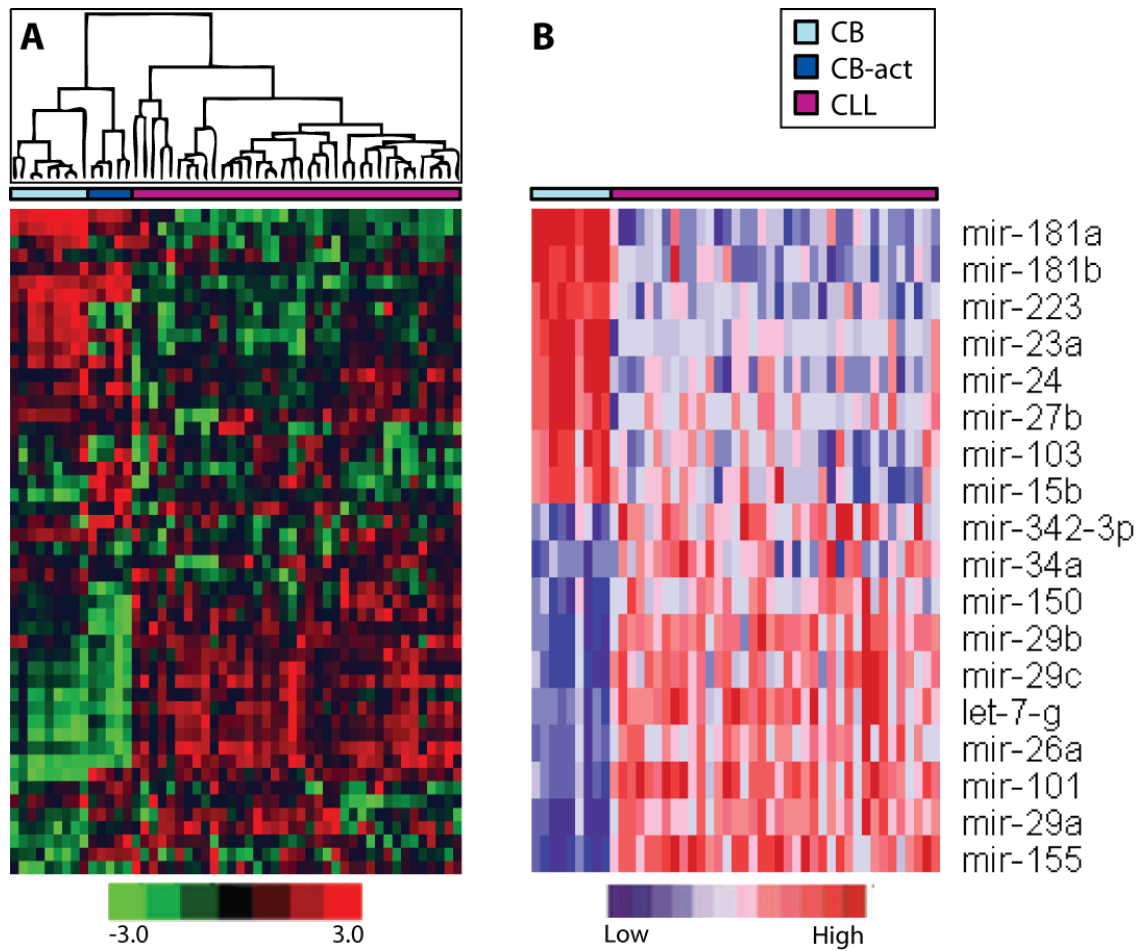
Associations between miRNA expression and clinical characteristics were assessed using the Fisher exact test for binary variables, and the Kruskal-Wallis test for variables with three or more categories. Time to first therapy was calculated as time from initial diagnosis to first therapy; patients not yet treated were censored at date last known alive. Time to first therapy was estimated using the method of Kaplan and Meier; the log rank test was used to assess associations with time to first therapy. Recursive partitioning was used to identify an optimal binary split for each miRNA, using the *rpart* package in R. *P*-values from recursive partitioning are not adjusted for the optimization of the method. Two-sided *p*-values are not adjusted to reflect multiple comparisons; *q*-values reflect adjustment for multiple comparisons using the false discovery rate of Benjamini and Hochberg, as implemented in the *q*-value package in R. A false discovery rate of 0.10 or smaller was the criterion for reporting significant differences in both clinical features and time to first therapy in this study.

## Results

### *Determinants of a CLL-specific miRNA expression signature*

We profiled miRNA expression across 38 highly purified CLL patient samples, 9 control B cell samples and 5 CpG activated B cell samples using the Luminex method [116]. CLL cells are characterized by expression of both CD5 and CD19 cell surface markers [118]. CD19 bead positive selection was used to purify CLL cells and greater than 97% CLL cell purity was verified by FACS analysis of CD5 and CD19 expression for all samples used in this study. Unsupervised hierarchical analysis clustered CLL samples based upon miRNA expression (Figure 3.1A). Comparative marker analysis in CLL samples compared with control B samples identified a CLL-specific miRNA signature consisting of upregulation of miR-342-3p, miR-34a, miR-150, miR-29a, b, and c, let-7g, miR-26a, miR-101, and miR-155, and downregulation of miR-15b, miR-103, miR-27b, miR-24, miR-23a, miR-223, and miR-181a and b. (Figure 3.1B).

Though most B cells are CD5-CD19+, specific subsets of B cells (e.g. peritoneal, tonsillar) are CD5+CD19+. To verify that miRNA expression profiling discerned differences between normal CD5-CD19+ B cells and CD5+ CD19+ CLL cells rather than between normal CD5-CD19+ and CD5+CD19+ B cells, control B cells from 4 different control donors were sorted into CD5- and CD5+ populations and RT-PCR analysis was performed. Our data indicate that miR-181a and miR-181b were downregulated in 6 CLL samples relative to both CD5- and CD5+ control B cell populations. Similarly, miR-29a, miR-150, and miR-155 were upregulated in CLL relative to both CD5- and CD5+ control B cell populations. This indicates that CD5+ expression status does not affect the interpretation of the changes in miRNA expression in these studies.



**Figure 3.1: miRNA expression accurately classifies control B cell, activated B cell, and CLL samples.** **A)** Heatmap of miRNA expression across 38 patient-derived CLL samples, 9 donor B cell samples, and 6 CpG activated donor B cell samples. miRNA expression is hierarchically clustered on the Y-axis and patient-derived CLL samples or control B cell donors are hierarchically clustered on the X-axis. The relative expression of miRNAs is depicted according to the color scale shown on the bottom. CB: donor control B sample; CB-act: donor B samples activated with CpG; CLL: CLL samples. **B)** Heatmap of miRNAs with differential expression in donor control B cells and CLL: Differentially expressed miRNAs were identified by comparative marker selection. MiRNAs are grouped by comparative marker score. The relative expression of miRNAs is depicted according to the color scale shown on the bottom.

### ***miRNA alterations identify an activated B cell phenotype in CLL cells***

A number of the miRNAs identified as part of the CLL specific signature overlap with miRNA expression signatures we observed in T-cell development and activation. Notably, the miR-26 and miR-29 families, together with miR-150 and let-7g are upregulated during maturation of T cells (Figure 2.1C). However, the most differentially expressed miRNAs in CLL are those we previously associated with murine T cell activation. miR-181a is the most significantly downregulated miRNA in CLL cells as compared to unactivated B cells, and has been shown to be downregulated after T cell activation (Figure 2.1B) [79], miR155 is strongly induced after T cell activation, and is the most significantly upregulated miRNA in CLL cells relative to unactivated B cells (Figure 2.1D and 3.1B). We thus hypothesized that CLL cells may share a common pattern of miRNA mediated gene regulation with activated B cells.

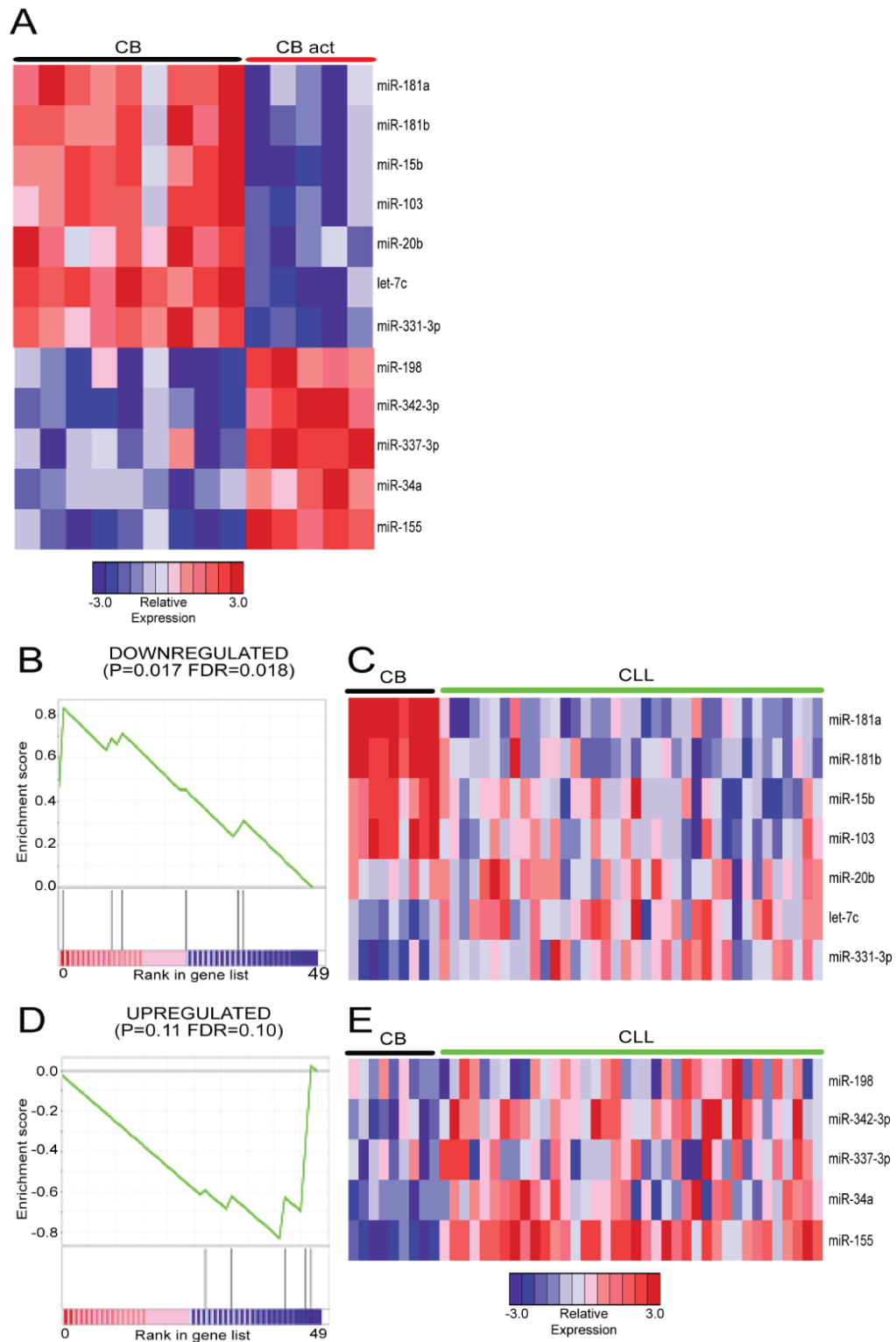
To systematically compare miRNA expression in CLL to the miRNA changes induced by B cell activation, we identified sets of miRNAs that were most significantly ( $p < 0.05$  after correction for multiple comparisons) upregulated or downregulated after untransformed B cell activation by CpG. The upregulated miRNAs were miR-34a, miR-198, miR-155, miR-337-3p and miR-342-3p and the downregulated miRNAs were let-7c, miR-15b, miR-20b, miR-103, miR-181a, miR-181b, and miR-331-3p (Figure 3.2 A). We then performed gene set enrichment analysis (GSEA) for these upregulated and downregulated miRNAs in CLL versus untransformed B cells (Figure 3.2 B-E). Four out of seven downregulated miRNAs (miR-15b, miR-103, miR-181a, and miR-181b) were expressed at lower levels in CLL, and five out of five upregulated miRNAs (miR-34a, miR-155, miR-198, miR-337-3p and miR-342-3p) were expressed at higher levels in CLL compared to untransformed B cells.

To independently verify our Luminex profiling data and confirm a link between the miRNA changes observed in CLL and B cell activation, we tested expression of B cell activation



**Figure 3.2: GSEA reveals a B cell activation miRNA signature in CLL.** **A)** Heatmap of B cell activation signature miRNAs comparing expression in unstimulated control B (CB) cells and CpG activated B cells (CB act). **B)** GSEA enrichment profile of downregulated B cell activation signature miRNAs in control B cells versus CLL cells. **C)** Heatmap of downregulated B cell activation signature miRNA expression in control B cells and CLL cells. **D)** GSEA enrichment profile of upregulated B cell activation signature miRNAs in control B cells versus CLL cells. **E.** Heatmap of upregulated B cell activation signature miRNA expression in control B cells and CLL cells.

**Figure 3.2 (Continued)**



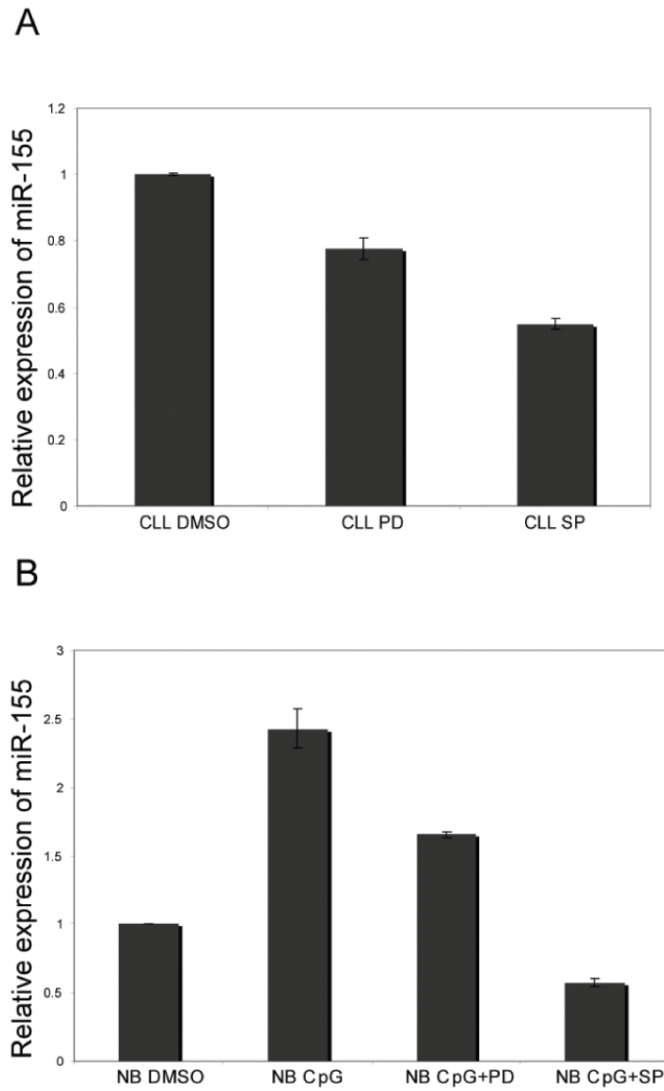
induced and CLL signature miRNAs in activated B cells and CLL cells by RT-PCR. We purified B cells from healthy donors and stimulated these cells with a variety of B cell activators including  $\alpha$ -IgM and CD40L (BCR and T cell-assisted co-stimulatory pathways), and LPS, CpG, or polyI:C Toll like receptor (TLR) pathways and examined miRNA expression (Summarized in Table 3.1) [98]. For the CLL signature miRNAs, we found that activation of control B cells led to reduced miR-23a, miR-23b, miR-24, miR-27b, miR-181a, miR-181b, and miR-223 (all downregulated in the CLL signature) and increased miR-155 (upregulated in the CLL signature), regardless of the mechanism of B cell activation. Most miRNAs demonstrated consistently altered expression regardless of the mechanism of B cell activation though the expression of the miR-29 family varied depending upon B cell activation. Whereas  $\alpha$ -IgM and CD40L upregulated miR-29a, LPS, CpG, or polyI:C downregulated miR-29a. In contrast,  $\alpha$ -IgM and CD40L downregulated miR-29b and miR-29c but LPS, CpG, or polyI:C upregulated miR-29b and miR-29c. miR-223 is increased in response to CD40L activation while decreased in response to other stimuli. Additionally, the expression of miR-26a was increased in CLL and only in B cells activated with CD40L but did not change in B cells activated with  $\alpha$ -IgM. These data suggest that particular CLL signature miRNAs are altered in response to specific B cell stimuli. In contrast, miR-150 was reduced during B cell activation, and upregulated in almost all the CLL samples tested (Figure 3.1 and Table 3.1). Thus, high expression of miR-150 may be CLL specific.

To mechanistically link altered miRNA expression in CLL with altered expression of miRNAs observed in B cell activation, we carefully examined the expression of one miRNA (miR-155) whose expression is increased in CLL and in activated B cells. The miR-155 gene is activated upon B cell stimulation and contains binding sites for the AP-1 transcription factor. B cell activation stimulates the JNK pathway, increases the levels of phospho-ERK, and then activates AP-1 [119]. We confirmed that treatment of CpG activated B cells with either JNK or MEK inhibitor decreased the expression of miR-155 (Figure 3.3A). CLL cells express high

**Table 3.1. Signature miRNA expression comparison between activated B cells and CLL cells relative to control B cells.**

miRNA list	B cell Activation		CLL
	IgM / CD40L	TLRs	
let-7g	↓	↓	↑
miR-23a	↓	↓	↓
miR-23b	↓	↓	↓
miR-24	↓	↓	↓
miR-26a	-/↑	↑	↑
miR-27b	↓	↓	↓
miR-29a	↑	↓	↑
miR-29b	↓	↑	↑
miR-29c	↓	↑	↑
miR-101	↓	↓	↑
miR-150	↓	↓	↑
miR-155	↑	↑	↑
miR-181a	↓	↓	↓
miR-181b	↓	↓	↓
miR-223	↓/↑	↓	↓

Relative expression of signature miRNAs in activated B cells and CLL cells was measured by RT-PCR. Up and down arrows indicate upregulation or downregulation of specific miRNAs, respectively, when compared to untransformed, control B cells. A parallel line indicates no change when compared to untransformed, control B cells. miR-26a is unaffected by IgM stimulation, but upregulated in response to other B cell activators. miR-223 is upregulated by CD40L stimulation, but downregulated by other B cell activators.



**Figure 3.3: Inhibition of miR-155 expression by MEK and JNK inhibitors.** **A.** Relative expression of miR-155 in CLL cells treated with MEK (PD98059; PD) and JNK (SP600125; SP) inhibitors. **B.** Relative expression of miR-155 in CpG-activated B cell treated with PD98059 and SP600125 inhibitors. Cells were cultured with DMSO as a control (DMSO).

steady state levels of miR-155, and treatment with JNK or MEK inhibitors decreases miR-155 expression (Figure 3.3 B). These data indicate common signaling pathways lead to altered miRNA expression in activated B cells and CLL cells.

***Signature miRNA alterations are associated with clinical features and time to first therapy***

Previous reports suggest that altered miRNA expression has prognostic value [120-122]. ZAP70 and IgV<sub>H</sub> mutational status are strongly associated with clinical outcome for CLL patients. Thus, comparative marker alignments were performed to identify miRNAs clustered by ZAP70 and IgV<sub>H</sub> status (Table 3.2 A and B). Out of 38 patients, we examined 14 ZAP70<sup>+</sup> patients and 14 IgV<sub>H</sub> unmutated patients. We found that miR-150 was upregulated in ZAP70<sup>+</sup> patients whereas miR-29c and miR-223 were upregulated in both ZAP70<sup>-</sup> and in IgV<sub>H</sub> mutated patients suggesting that decreased levels of miR-150 and increased levels of miR-29c and miR-223 may be associated with better clinical outcomes. Our data do not indicate a statistically-significant correlation between miR-29c and miR-223 with any established cytogenetic abnormalities used to distinguish CLL patient sub-populations, such as 17p, 11q and 13q deletion and trisomy 12 (data not shown). Additionally, miR-92a was upregulated in ZAP70<sup>-</sup> patients and let-7g was upregulated in IgV<sub>H</sub> mutated patients (Table 3.2 A and B).

We also examined miRNA expression across CLL cells for association with time to first therapy in these patients. Using recursive partitioning analysis for finding optimal cut points for miRNA expression, we examined time to first therapy. We identified two miRNAs (miR-29c and miR-223) with *q*-values of 0.07 associated with an optimized binary split (Kaplan-Meier curves and the optimal splits are indicated (Figure 3.4)). CLL patients with lower expression levels of these miRNAs demonstrated shorter time to first therapy compared to CLL patients who did not demonstrate this pattern of miRNA expression. Decreased expression of these two miRNAs was

also associated with ZAP70<sup>+</sup> and IgV<sub>H</sub> unmutated patients (Table 3.2 A and B) implying poor prognosis.

**Table 3.2 A. miRNAs correlated with ZAP70 status.**

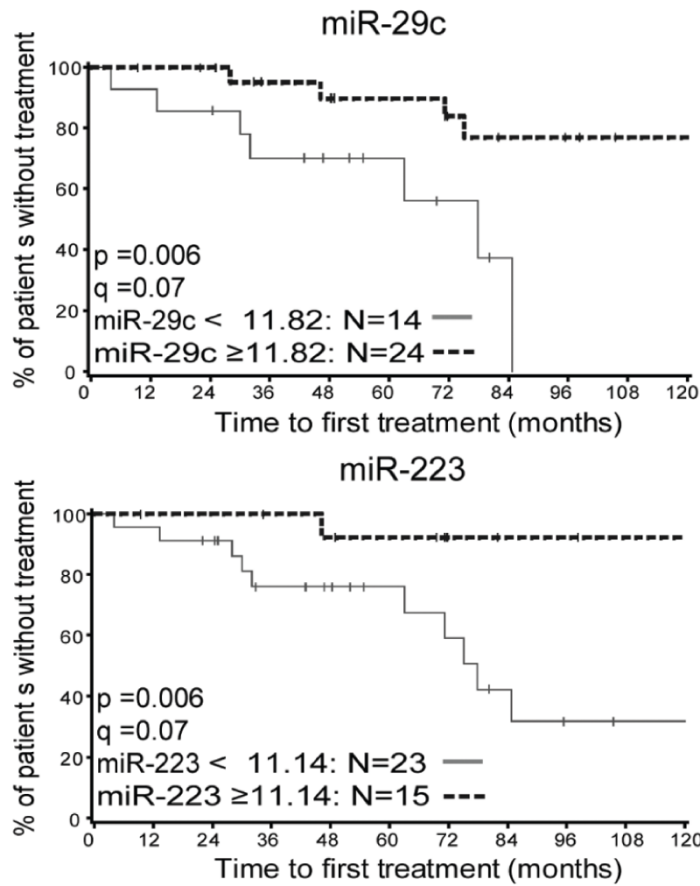
miRNAs	ZAP70					
	Negative		Positive		<i>p</i>	<i>q</i>
	N	Median (Min, Max)	N	Median (Min, Max)		
miR-150	22	13.67 (13.16, 14.89)	14	14.05 (13.64, 15.05)	0.009	0.09
miR-223	22	11.28 (10.08, 11.98)	14	10.81 (9.47, 11.23)	0.005	0.08
miR-29c	22	12.13 (10.99, 12.53)	14	11.62 (10.90, 12.13)	0.006	0.09
miR-92a	22	11.10 (10.81, 11.72)	14	10.95 (10.40, 11.24)	0.004	0.08

**Table 3.2 B. miRNAs correlated with IgV<sub>H</sub> status.**

miRNAs	IgV <sub>H</sub> mutation					
	Mutated		Unmutated		<i>p</i>	<i>q</i>
	N	Median (Min, Max)	N	Median (Min, Max)		
let-7g	17	12.01 (11.54, 12.33)	14	11.79 (11.22, 12.34)	0.005	0.07
miR-223	17	11.29 (10.62, 11.98)	14	10.65 (9.47, 11.07)	0.0005	0.02
miR-29c	17	12.20 (11.25, 12.53)	14	11.49 (10.90, 12.25)	0.004	0.06

Wilcoxon rank sum tests were used to identify differences on miRNA expression pattern between patients with different clinical features such as ZAP70 status (3.2 A), and IgV<sub>H</sub> status (3.2 B). *p*-value and *q*-value for each miRNA are shown. N: patient number





**Figure 3.4: The expression of miR-29c and miR-223 is associated with time to first therapy in CLL.** A shorter time from diagnosis to initiation of first therapy is significantly associated with reduced expression of miR-29c and miR-223. A recursive partitioning approach identifies the optimal split for each miRNA: miR-29c expression at 11.82 or lower is associated with shorter time to first therapy,  $q = 0.07$ . miR-223 expression at a level of 11.14 or lower is associated with shorter time to first therapy,  $q = 0.07$ .

## Discussion

miRNA expression profiling has been used to identify signatures that classify CLL [108-110, 123]. Interestingly, none of these published signatures are identical. The reasons for the different miRNA signatures are unknown. Different methods and different miRNA analysis platforms may partly explain the different signatures identified by each of these groups. Calin et al. profiled 94 CLL patient samples using a miRNA microarray but did not purify CLL cells to homogeneity [108, 109]. Fulci et al. profiled 56 CLL patient samples by cloning and SYBR-green-based RT-PCR [110]. Zanette et al. profiled miRNA expression in 9 CLL patients and 6 healthy donors using TaqMan-based miRNA assays [111]. In addition, CLL is a heterogeneous disease and it is possible that different patient populations have unique miRNA expression patterns. Zhang et. al. [112] demonstrated CLL cells are more like memory B cells based on miRNA profiling, whereas our miRNA profiling data associated CLL cells with miRNA changes induced during early activation of B cells.

The uniformity of miRNA expression changes we observed in CLL patient samples relative to B cells is striking (Figure 3.1). Though the directionality of changes in expression is consistent for these particular miRNAs, the magnitude of changes varies considerably for these particular miRNAs. Indeed, some miRNAs demonstrate expression variance over several orders of magnitude. This variability in expression of these miRNAs may be related to the heterogeneous clinical course. Another striking finding is that many CLL signature miRNAs demonstrate similar trends in activated B cells regardless of the mechanism of activation.

However, certain CLL signature miRNAs are differentially regulated by specific B cell activators. We show that miR-29b and miR-29c are downregulated by IgM and CD40L activation but are upregulated with TLR activation. Published reports on miRNA expression in naïve, germinal center, memory, and plasma B cell populations indicate that these miRNAs are also differentially regulated during B cell differentiation [112, 124]. miR-29c is reduced in germinal

center B cells [112] and in centroblasts [124] but is increased in memory B cells [112]. The pattern of miR-29 family member expression may be affected by the duration of B cell activation or the simultaneous stimulation of multiple activation pathways during the germinal center reaction. Interrogating the causes and consequences of altered miRNA expression in CLL cells in common with and distinct from altered miRNA expression in activated B cells will improve our understanding of this disease.

We identify a correlation between miR-29c and miR-223, negative clinical markers, and shorter times to first therapy. These observations are consistent with previous observations that have associated reduced levels of these two miRNAs with ZAP70, IgV<sub>H</sub> unmutated status, and poor clinical outcomes. [109, 110, 122, 125]. In addition, CLL patients expressing CD38 tend to have higher expression of activated cellular markers, including CD27, CD62L, CD69 and Ki-67, and this activation status is correlated with poor clinical outcomes [126, 127]. Our study integrates these two observations. We found that clinically relevant alterations in miR-29c and 223 are part of a larger pattern of miRNA changes consistent with B cell activation

miR-29c and miR-223 co-vary with other clinical parameters and thus may not have independent prognostic value, but these miRNA changes may have biological function in CLL. BCR activation led to downregulation of miR-29c and miR-223 and ZAP70 is associated with increased BCR signaling in CLL [128]. Although the kinase domain of ZAP70 is not required for BCR pathway stimulation in CLL, the scaffolding function of ZAP70 acts as an adaptor that clusters and thereby increases signaling through BCRs in CLL [128, 129]. It is likely that the association we observed with ZAP70+ CLL and lower miR-29c and miR-223 is due to enhanced BCR signaling mediated by ZAP70 [128]. Reduced levels of miR-29c have been shown to de-repress expression of MCL-1 and TCL-1, known oncogenes implicated in the CLL  $\alpha$ -apoptotic defect [120, 121, 123]. This downregulation of miR-29c may play an important role in the poor clinical outcomes associated with ZAP70+ CLL.

Other miRNA changes consistent with B cell activation may also have an important role in CLL oncogenesis. miR-155 is strongly upregulated in all CLL samples tested as compared to control B cells, is increased in multiple cancers [130-132], and promotes B cell lymphomagenesis [133]. Conversely, miR-181a and miR-181b are strongly downregulated in all CLL samples tested. PI-3K is a predicted target of miR-181a and miR-181b [21] and the PI3K pathway has been implicated in the apoptotic defect in CLL cells [134]. These data suggest that miR-155 has an oncogenic function, whereas miR-181a and miR-181b have a tumor suppressive function in CLL. Analysis of changes in miRNA expression and their target genes during B cell activation and CLL oncogenesis will provide insights into the physiological roles of these miRNAs. Understanding the roles of miRNAs in the dysregulated gene networks that underlie the pathology of CLL could enable the application of miRNA-based therapeutics for this common leukemia.

## **Chapter 4**

**Dynamic microRNA expression during CD8 T cell activation:**

**a role for miR-31**

## Abstract

Cytotoxic CD8 T cells are important mediators of the adaptive immune response. However, miRNA expression signatures and the roles of individual miRNAs during the CD8 T cell response are poorly characterized. We demonstrate dynamic changes in miRNA expression during early CD8 T cell activation, including decreased expression of miR-150, miR-26, miR-29 and miR-30, and increased expression of miR-155, miR-31, miR-146 and the miR-17-92 cluster. We show that miR-31 is strongly induced by calcium/Calcineurin-dependent processes during T cell activation, with highest induction in CD8 T cells. miR-31 expression is maintained in CD4 and CD8 memory T cell populations generated *in vitro* and *in vivo*. We identify miR-31 targets in CD8 T cells, including *Lats2*, *Ppp6c*, *Stk40*, *Sh2d1a*, *Ilf3*, and *Cdkn1a*, and propose a model whereby miR-31 induction primes CD8 T cells for activation by repressing inhibitors of multiple activation-induced pathways.

## Introduction

CD8 T cells are essential effectors of the adaptive immune response to viruses and intracellular pathogens capable of inducing the death of infected somatic or tumor cells. The CD8 T cell response is characterized by rapid, dramatic shifts in cellular function and gene expression. Naïve CD8 T cells are quiescent, with low metabolic activity, little to no cell division, and virtually no cytotoxic activity. After TCR engagement by a cognate peptide-MHC complex, these cells expand more than 1000 fold, and rapidly acquire a panel of effector functions, including cytolytic activity and cytokine production [135, 136]. Underlying this transition are changes in multiple gene networks affecting survival, proliferation, effector function, and cell trafficking. Microarray mRNA expression profiling has been used to discover the genes and pathways which drive CD8 T cell differentiation and activation [137, 138]. Given the important roles of miRNAs in developmental transitions, it is likely that they are also involved in CD8 T cell activation. Strong evidence for this role comes from conditional deletion of Dicer, an RNaseIII enzyme that is required for the biogenesis of almost all miRNAs. Deletion of Dicer during T cell development in the thymus leads to a dramatic reduction in CD8 T cell levels in the periphery [67, 139]. Loss of Dicer in mature CD8 T cells leads to enhanced activation and proliferation *in vitro*, but a dramatically reduced CD8 T cell effector response *in vivo* [140]. These results clearly show that miRNAs play important roles in CD8 T cell development and function.

Two groups have profiled miRNA expression in CD8 T cells that were induced to differentiate towards an effector or central memory phenotype *in vitro* by culture with antigen and IL-2 or IL-15 [141]. Almanza et al. compared miRNA expression by microarray in differentiated subsets after antigen stimulation [142]. They identified miRNAs with differential expression in memory subsets, including miR-150, miR-155, and members of the let-7 family, and proposed a model whereby high miR-150 and low miR-155 promotes development of central memory cells.

Wu et al. profiled miRNAs in naïve, effector and central memory subsets by small RNA cloning and microarrays [143]. They report a global downregulation of the majority of miRNAs during transition from naïve to effector memory subset, with a more modest downregulation during transition from naïve to central memory subset. Wu et al. report upregulation of only a few miRNAs in memory subsets relative to naïve subsets, most notably miR-21. The paradigm of lower global miRNA activity in CD8 T cells is consistent with other changes in gene regulation seen during T cell activation. Deep sequencing of transcripts has revealed that proliferating T cells tend to use more proximal polyadenylation sites, leading to shorter 3'UTRs [144]. As most miRNA target sites are in the 3'UTRs, shortening of 3'UTRs may synergise with lower miRNA levels and lead to a general loss of miRNA-mediated repression. Given the limited number of miRNA profiles available for the process of CD8 T cell activation and lack of consensus regarding the miRNA activation signature in these cells, additional studies of the changes in miRNA expression are needed to determine the roles of miRNAs in the CD8 T cell response.

Individual miRNAs have been shown to play important roles in T cell activation. miR-146a and miR-155 are strongly induced after activation of both innate and adaptive immune cells, including CD8 T cells. Both of these miRNAs participate in a negative feedback loop regulating NF- $\kappa$ B signaling, and are required for optimal Treg function [145-147]. In addition, both miR-146a and miR-155 are involved in regulation of cytokine signaling. miR-146a downregulates type 1 interferon (IFN) signaling by repressing the key signaling intermediates Traf6, Irak1/2 and Stat1 [51, 145]. In contrast, miR-155 upregulates cytokine signaling by repressing the Socs1 suppressor of cytokine signaling, and promotes Th1 type development [44, 147]. The downregulation of miRNAs expressed in naïve T cells can also play important roles in T cell activation. For instance, miR-29c is downregulated in activated NK cells, CD4 T cells, and CD8 T cells relative to naïve T cells. miR-29 inhibits interferon- $\gamma$  production either directly by repressing the interferon- $\gamma$  mRNA itself, or indirectly by repressing upstream transcription factors



essential for interferon- $\gamma$  expression [148, 149]. The release of interferon- $\gamma$  from inhibition by miR-29c after activation promotes secretion of this important cytokine.

However, the roles of the majority of the miRNAs expressed in T cells are still unknown. In this study, we profile miRNA expression during early CD8 T cell activation, and identify miR-31 as novel activation-induced miRNA in mouse T cells. miR-31 is induced after TCR stimulation in a calcium-dependent manner, and continues to be expressed in memory cell populations *in vitro* and *in vivo*. We identified mRNA targets of miR-31 in primary T cells, which include pro-apoptotic factors and negative regulators of cyclin, NF- $\kappa$ B and MAPK signaling. We propose a model whereby miR-31 induction primes cells for activation by promoting T cell survival, activation, and proliferation.

## **Materials and methods**

### ***Mice***

Male c57bl/6 and OT-I TCR transgenic mice between 5-10 weeks of age were purchased from Taconic Farms (Hudson, NY, USA). Foxp3-GFP reporter mice were obtained from Dr. Vijay Kuchroo.

### ***Isolation of immune cell subsets***

CD8 T cells were isolated by negative selection using EasySep beads (Stemcell technologies, Vancouver, BC, Canada.). B cells and NK cells were isolated from mouse spleen and peripheral lymph nodes by FACS. B cells were sorted as CD19<sup>+</sup>/B220<sup>+</sup> and NK cells as DX5<sup>+</sup>/CD3<sup>-</sup>. Endogenous activated, memory, and Treg T cell populations were isolated from spleen and PLN by FACS. Naïve and memory cells were sorted from CD4 and CD8 as: naïve (CD4/8<sup>+</sup> CD62L<sup>+</sup> CD44<sup>+</sup> CD25<sup>-</sup>); central memory (CD4/8<sup>+</sup> CD62L<sup>+</sup> CD44<sup>+</sup> CD25<sup>-</sup>); effector memory (CD4/8<sup>+</sup> CD62L<sup>-</sup> CD44<sup>+</sup> CD25<sup>-</sup>). Treg cells were sorted as CD4<sup>+</sup> CD25<sup>+</sup> GITR<sup>+</sup>. *In vivo* activated CD8 cells were sorted as CD8<sup>+</sup> CD25<sup>+</sup>. Foxp3GFP<sup>+</sup> and Foxp3GFP<sup>-</sup> CD4 T cells were isolated from Foxp3GFP reporter mouse splenocytes stained with  $\alpha$ -CD4. Anti B220, CD19, CD25, DX5 and CD3 antibodies were obtained from Biolegend (San Diego, CA, USA). Anti CD4, CD8, CD62L, CD69 and CD44 antibodies were obtained from eBioscience (San Diego, CA, USA)

### ***Cell culture and activation***

Cells were cultured in RPMI-1640 Medium supplemented with 10% fetal calf serum, 50 units/ml Penicillin-Streptomycin, 10 mM HEPES, 2 mM L-glutamine, and 55  $\mu$ M  $\beta$ -mercapto-ethanol (all from GIBCO, Carlsbad, CA, USA) in a 37°C incubator with 5% CO<sub>2</sub>. T cells were activated with 10  $\mu$ g/ml plate bound  $\alpha$ -CD3 with or without 2  $\mu$ g/ml soluble  $\alpha$ -CD28 antibodies

(eBioscience, San Diego, CA, USA) or 500 nM ionomycin. Cyclosporin A was added at 250 ng/ml. NK cells were activated with PMA (5 ng/ml) and ionomycin (500 nM). B cells were activated with 20 ng/ml IL-2, 1 µg/ml α-IgM (Southern Biotech, Birmingham, AL, USA) and 3 µg/ml CpG 2006. Murine T hybridoma cells were isolated as described [150].

### ***Luminex profiling of miRNA expression***

MiRNA expression was profiled as described in Chapter 2.

### ***MiRNA-specific RT-PCR assay***

MiRNA specific cDNA synthesis from total RNA was carried out using MicroRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) miRNA-specific RT-PCRs were performed using Taqman miRNA assays according to the manufacturer's protocol (Applied Biosystems, Carlsbad, CA, USA). miRNA expression was calculated using the  $\Delta\Delta C_t$  method with using miR-16 as an expression standard.

### ***NFAT binding site prediction and chromatin immunoprecipitation***

Computational prediction of NFAT binding sites was performed using the Lever algorithm [151]. NFAT chromatin immunoprecipitation was performed as described. [152]. Briefly, chromatin fragments from untreated and α-CD3/CD28 treated (24 hrs) primary CD8 T cells were immunoprecipitated with 6 µg of α-NFAT antibody sc-5499 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). DNA extraction was performed using a Qiagen purification kit. Site enrichment was analyzed by RT-PCR using the following primers:

forward: 5'-TATAGGGGATGGTTTGGGAAT-3'

reverse: 5'-ACCAAGAGCTTTTACACGTCCT-3'

### ***Lentiviral infection, microarray profiling, and miR-31 target gene prediction***

SiRNA containing lentiviral vectors against murine Lats2, Ppp6c, Stk40, and Sh2d1a were obtained from the RNAi Consortium and subcloned into the Plko.3G vector. miR-31 precursor sequence was generated by primer extension, and directly cloned into the Plko.3g vector using EcoRI/PacI enzyme digest. Empty Plko.3g constructs were used as controls for infection. Cloned mir-31 hairpin sequence is:

TGCTCCTGTAACTCGGAAGTGGAGAGGAGGCAAGATGCTGGCATAGCTGTTGAACTG  
AGAACCTGCTATGCCAACATATTGCCATCTTTCCTGTCTGACAGCAGCTTGGCTACCT  
C

CD8 T cells were purified from OT-I mice (Taconic, Hudson, NY, USA) by negative selection using EasySep beads (Stemcell technologies, Vancouver, BC, Canada). Purified T cells were cultured for 2 days in RPMI-10 with 100ng/ml IL-15 and 1 ng/ml IL-7 (R&D biosystems Minneapolis, MN, USA). Cells were infected with lentiviral vectors at a MOI of 10:1 by spin infection in Retronectin coated plates (Takara Bio, Otsu, Shiga, Japan). After infection, cells were cultured for 3 additional days in RPMI-10 with 10 ng/ml IL-2 (R&D biosystems Minneapolis, MN, USA). GFP<sup>+</sup> cells from 4 independent cultures per condition were then sorted by FACS and RNA isolated with Trizol followed by cleanup with RNeasy columns (Qiagen Valencia, CA, USA). 10 µg RNA/condition were profiled on Affymetrix Mouse Genome 430 2.0 Arrays. Array data analysis was performed using DCHIP, followed by Significance Analysis of Microarrays to isolate differentially expressed genes [153, 154]. A master list of miR-31 target genes was generated using the union of mouse miR-31 target predictions from Targetscan, Starbase, Rna22, Pictar and Miranda [155-159].

### ***Luciferase Reporter Assay***

3' UTR sequences containing predicted target sites and at least 500 base pairs of flanking sequence were cloned downstream of a firefly luciferase reporter in the pcDNA3.1 mammalian expression vector. 293T cells were transfected in a 96-well format with 20 µg Firefly reporter, 20 µg control pcDNA3.1 renilla luciferase reporter, and 200 µg of control or miR-31 Plko.3g vector. Luciferase activity was quantified at 24 hours with DualGlo reagents (Promega, Madison, WI, USA)

### **Western blotting**

Cells were lysed in RIPA buffer with P8340 protease inhibitor cocktail (Sigma St. Louis, MO, USA), 100 µM phenylmethanesulfonylfluoride, and 10 mM sodium orthovanadate. An equal number of cells per lane were run on NuPage 12% Bis-Tris gels (Invitrogen) and transferred to polyvinylidene fluoride membranes using a mini-trans blot apparatus (BioRad, Hercules CA, USA) according to the manufacturer's instructions. Antibodies to Sh2d1a (Fl-128) and CD3 (m20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

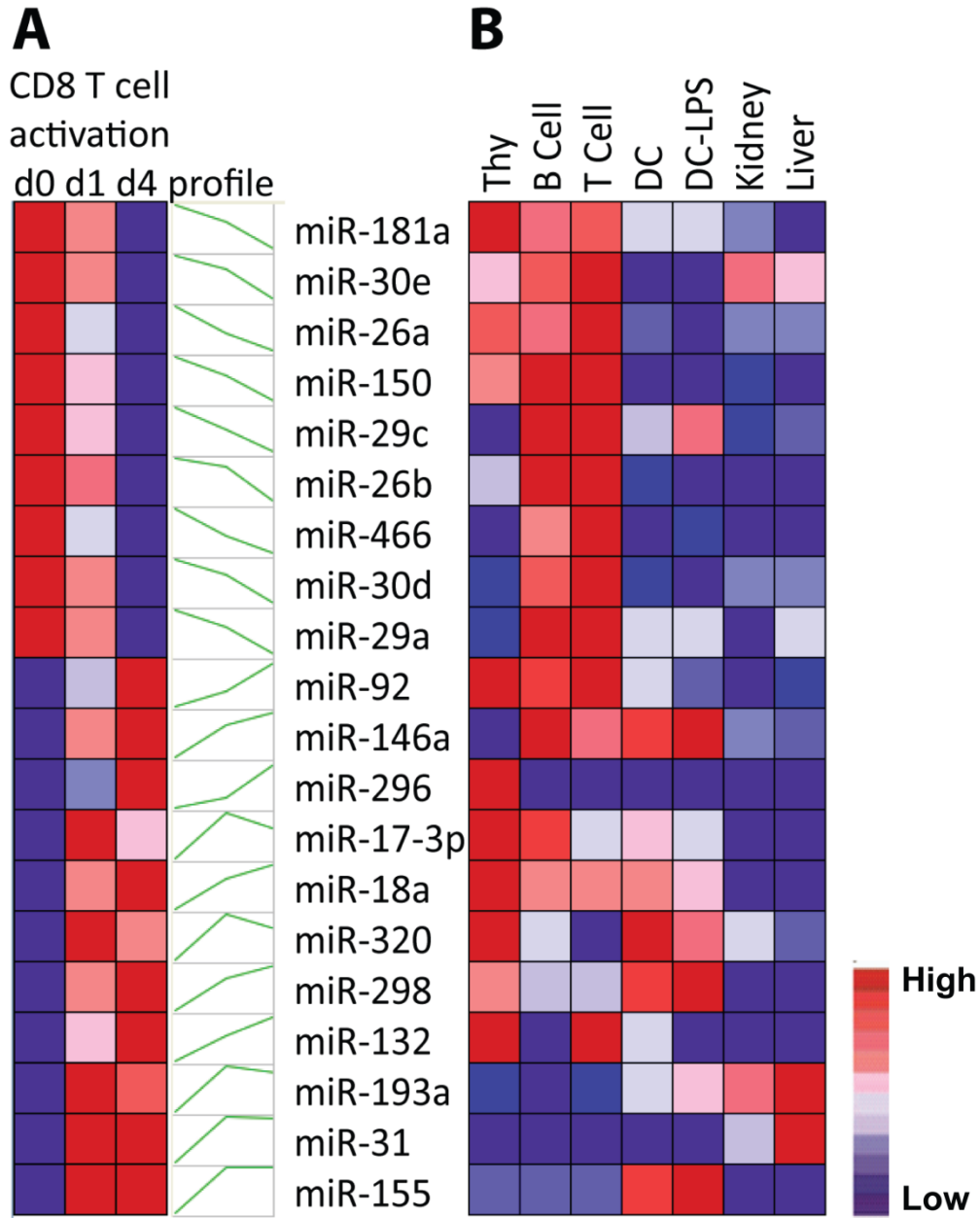
Antibodies to Ppp6c and Ilf3 were obtained from Abcam (Cambridge MA, USA). Antibodies to alpha-tubulin, ERK1/2, phospho-ERK1/2, phospho-Mek, and phospho-p38 were obtained from Cell Signal (Danvers, MA, USA).

## Results:

### *Early CD8 T cell activation induces dynamic changes in the expression of many miRNAs*

Current data on miRNA expression in CD8 T cells is inconclusive and has focused on miRNA expression in *in vitro* generated memory subtypes. However, many of the most dramatic changes in gene expression and cellular phenotype occur early after T cell activation. To determine which miRNAs participate in T cell development and activation, we used a Luminex bead-based system to profile murine miRNA expression in developing thymocytes, mature T and B cells, CD8 T cells at 1 and 4 days post-activation, bone marrow-derived dendritic cells (DC), and control kidney and liver tissue perfused to remove contaminating lymphocytes. Profiling data indicate that expression of a large number of miRNAs is altered during early CD8 T cell activation (Figure 4.1A) and reveal a novel dynamic regulation of miRNAs during early CD8 T cell activation.

miRNA alterations include the downregulation of miR-181a, miR-30 (e and d), miR-26 (a and b), miR-150, miR-29 (a and c) and miR-466 as well as the upregulation of miR-92, miR-146a, miR-296, miR-17, miR-18a, miR-320, miR-298, miR-132, miR-193a, miR-31 and miR-155. In general, miRNA downregulation was more pronounced at 4 days post-activation, whereas many of the upregulated miRNAs were near peak values at 1 day post-activation. The slower detection of downregulation is likely due to long miRNA half-lives and thus the requirement for miRNA turnover and dilution through cell division to remove pre-existing miRNAs. A second general feature of CD8 T cell activation is the concurrent regulation of multiple members of the same miRNA family, similar to what we observed during T cell development. Multiple members of the miR-30, miR-26, and miR-29 families of miRNAs are concurrently downregulated. Given



**Figure 4.1: Signature changes in miRNA expression during early mouse CD8 T cell activation from Luminex profiling. A)** miRNAs induced or suppressed at least 2.5 fold after CD8 T cell activation. miRNAs are ranked by fold change after activation. **B)** Expression of signature miRNAs in other mouse tissue types, including total thymocytes (Thy), B cells, T cells, bone marrow derived DC, LPS-stimulated DC, kidney, and liver.

the overlapping mRNA targets of miRNA family members, it is likely that reduction in the levels of multiple family members is required for maximum functional impact.

Some miRNAs we identify as being altered during CD8 T cell activation have known functions in the immune response. Among the downregulated miRNAs, the miR-26 and miR-29 family members have been shown to repress cytokine genes [148, 149, 160, 161], and miR-181a regulates sensitivity of TCR to triggering by repressing inhibitory phosphatases [79]. The upregulated miRNAs miR-17, miR-92, and miR-18a are derived from the miR-17-92 cluster on chromosome 13. This miRNA cluster is essential for B cell development, and ectopic expression is associated with cellular proliferation, hyperactivation and autoimmunity [162, 163]. As previously described, miR-146a and miR-155 play important roles in modulating the immune response. In general, the changes in miRNA expression we observe during early CD8 T cell activation prime the CD8 T cell effector activity by decreasing negative regulators of cytokine secretion and increasing positive regulators of general cytokine signaling, proliferation, and activation. Simultaneously, by reducing the sensitivity of CD8 T cells to TCR stimulation and inflammatory cytokines through downregulation of miR-181a and upregulation of mir-146a, miRNAs are exerting a negative feedback effect to limit the immune response.

However, the roles of many of the altered miRNAs detected during early T cell activation remain unknown. In particular, the second most upregulated miRNA in our profile, miR-31, does not have a known role in CD8 T cells. In contrast to other upregulated miRNAs with known functions in both T cell and innate immunity, miR-31 is not expressed in unstimulated or LPS stimulated bone marrow DCs (Figure 4.1B). Given that this miRNA displayed strong upregulation after activation and a strong T cell expression bias, we decided to investigate miR-31 in more detail.



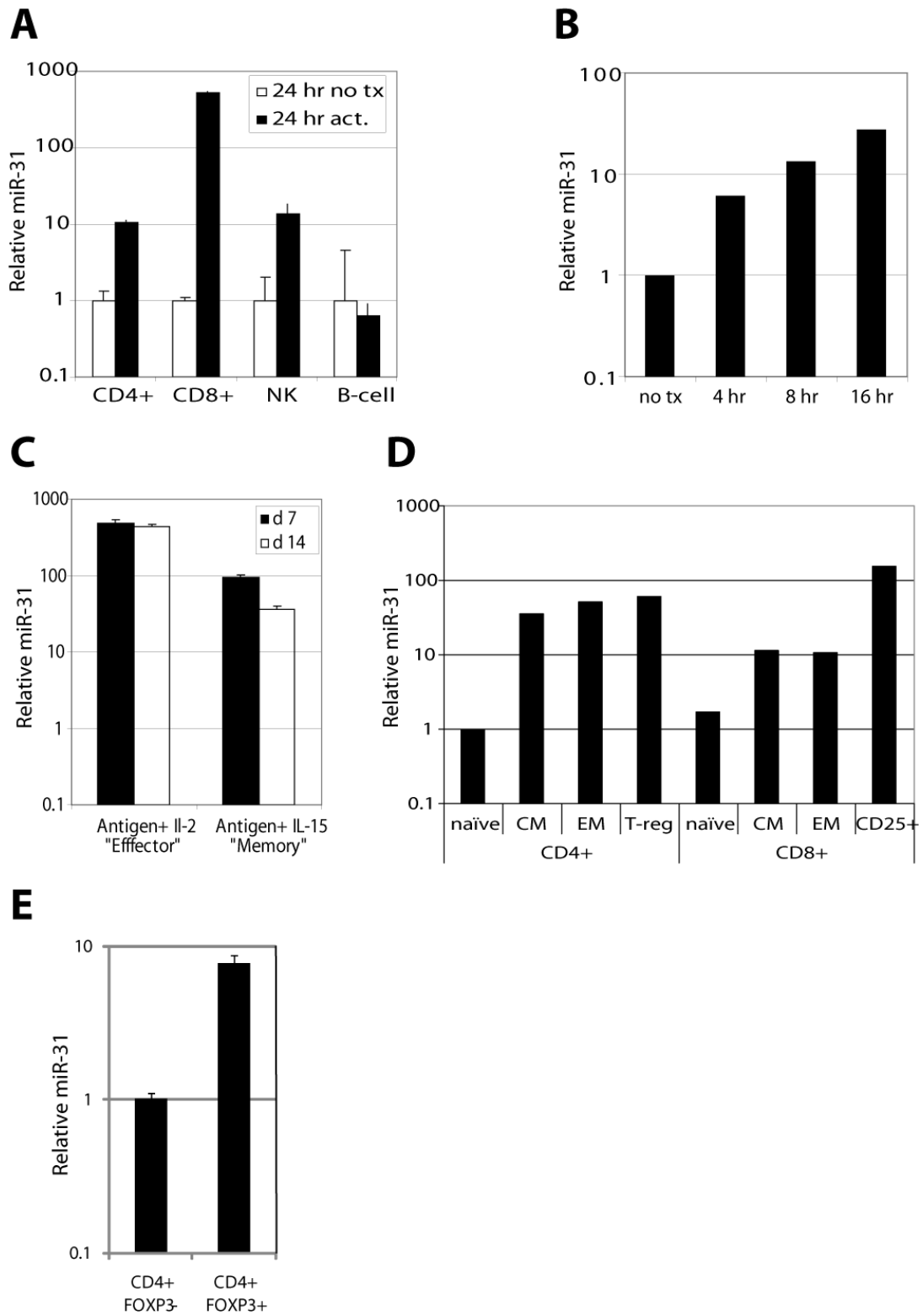
***miR-31 is expressed in activated and memory T cells and NK cells, but not in B cells.***

To determine if miR-31 induction is specific to T cells, we measured miRNA induction in other related immune cell types by RT-PCR (Figure 4.2A). Consistent with our Luminex profile, we observed strong upregulation of miR-31 in CD8 T cells after  $\alpha$ -CD3/28 stimulation. We also observed upregulation of miR-31 in CD4 T cells stimulated by  $\alpha$ -CD3/28 and in NK cells stimulated by PMA/ionomycin, although the magnitude of upregulation was significantly lower compared to that observed in CD8 T cells. Interestingly, we did not observe an increase in miR-31 expression in B cells after activation with  $\alpha$ -IgM antibody and CpG oligonucleotides. These data suggest that miR-31 plays a role in T cell specific processes.

Time course experiments revealed that an increase in mature miR-31 is detectable as early as 4 hours after CD8 T cell activation (Figure 4.2B). To determine if miR-31 expression was limited to early activation or persisted in differentiated CD8 T cell subsets, we tested miR-31 expression in memory T cells generated *in vitro* and *in vivo*. TCR transgenic CD8 T cells can be induced to differentiate *in vitro* towards effector memory or central memory phenotypes by antigen stimulus followed by culture with IL-2 or IL-15 cytokines [141]. We generated effector and central memory cells *in vitro* from OT-I CD8 TCR transgenic T cells, confirmed by FACS surface marker expression of CD62L<sup>-</sup> CD44<sup>+</sup> (effector memory) and CD62L<sup>+</sup> CD44<sup>+</sup> (central memory), and tested miR-31 expression at multiple time points (Figure 4.2C). miR-31 expression was maintained at very high levels during *in vitro* differentiation into effector cells compared to naïve T cells. In contrast, miR-31 levels progressively declined during *in vitro* differentiation into central memory cells, although expression levels remained higher than that seen in naïve cells. While cytokine signaling may play a role in maintaining miR-31 levels, it is not sufficient for miR-31 induction, as treatment of CD8 T cells with IL-2, IL-7, or IL-15 cytokines without TCR stimulation did not alter miR-31 expression (data not shown). These data indicate that CD8 T cell differentiation state regulates the long-term expression of miR-31 after antigen stimulation.

**Figure 4.2: miR-31 expression depends on cell type and differentiation state.** **A)** Relative miR-31 levels 24 hours after activation of CD4 or CD8 T cells with  $\alpha$ -CD3/CD28, NK cells with PHA/ionomycin, or B cells with  $\alpha$ -IgM/CpG. **B)** Relative miR-31 levels after stimulation of CD8 T cells for 0 hours (no tx), 4 hr, 8 hr, or 16 hours with  $\alpha$ -CD3/CD28. **C)** miR-31 expression in antigen-stimulated OT-I CD8 T cells cultured with the indicated cytokines to induce *in vitro* differentiation into effector and central memory phenotypes. miR-31 expression is given relative to that in OT-I cells prior to stimulation. **D)** miR-31 is expressed at elevated levels in both CD4 and CD8 central memory (CM), effector memory (EM), CD4 CD25 GITR regulatory (Treg), and CD8 CD25 *in vivo* activated T cell populations. **E)** miR-31 expression in Foxp3-GFP<sup>+</sup> and Foxp3-GFP<sup>-</sup> CD4 T cells from Foxp3-GFP reporter mice.

**Figure 4.2 (Continued)**



To confirm that the miR-31 expression we observed during *in vitro* differentiation is representative of *in vivo* expression patterns, we measured expression of miR-31 in sorted populations of naïve, effector memory, and central memory populations of CD4 and CD8 T cells, as well as CD4 regulatory T cells (Treg) and *in vivo* activated CD25<sup>+</sup> CD8 T cells (Figure 4.2D). Endogenous CD8 effector memory and central memory populations expressed elevated levels of miR-31, with peak expression seen in circulating CD25<sup>+</sup> CD8 T cells. The expression pattern of *in vivo* activated CD8 and central memory T cells closely paralleled the expression patterns we observed *in vitro*, with peak expression in activated T cells and maintenance of lower levels in central memory T cells. However, expression of miR-31 was not higher in effector memory compared to central memory CD8 T cells *in vivo*, suggesting that the IL-2 driven *in vitro* model of CD8 differentiation does not perfectly recapitulate *in vivo* differentiation patterns. Notably, similarly elevated miR-31 expression was also detected in CD4<sup>+</sup> memory populations and in CD4<sup>+</sup> CD25<sup>+</sup> GITR<sup>+</sup> Treg. The expression of miR-31 in Treg was confirmed in CD4 cells expressing the Foxp3-GFP reporter (Figure 4.2E). These results indicate that increased miR-31 expression is a feature of memory cell differentiation in both CD4 and CD8 subsets.

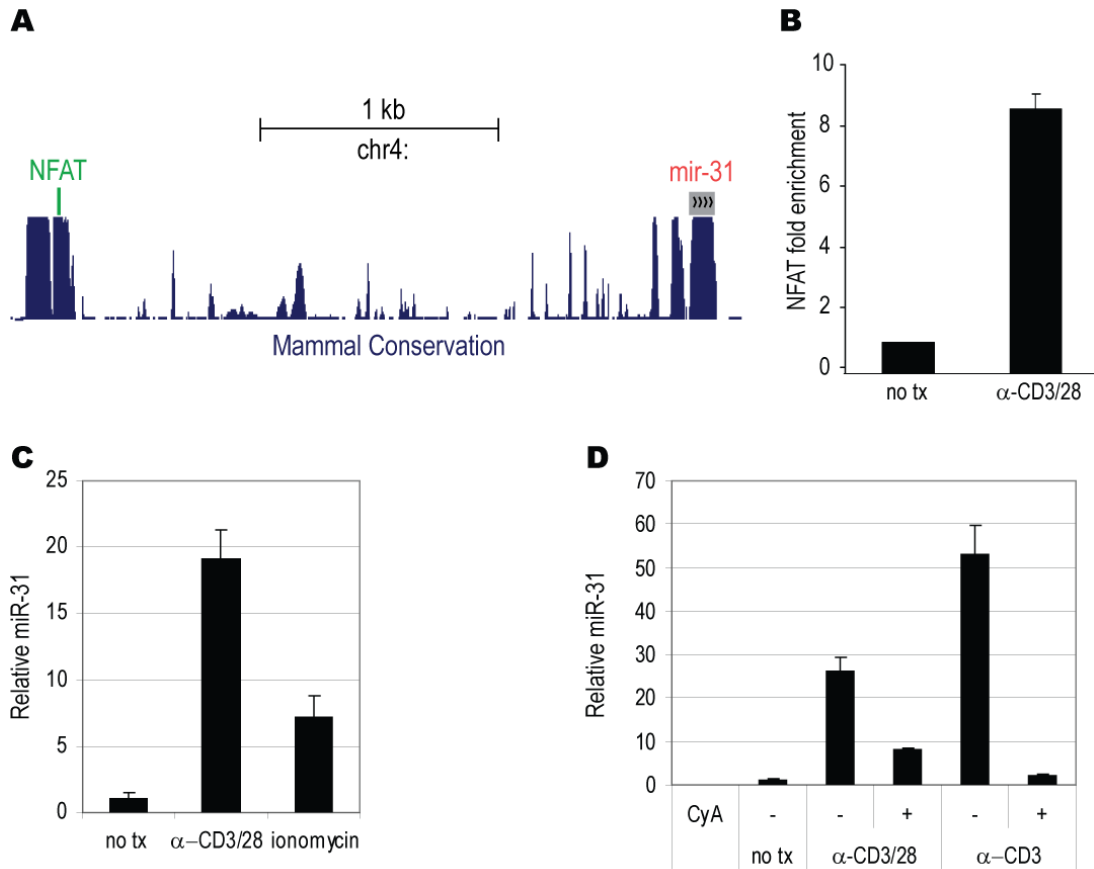
### ***miR-31 expression during T cell activation is regulated by calcium and calcineurin signaling***

Research into the transcriptional regulation of miRNA expression has lagged behind the rest of the miRNA field. One important factor which complicates studies of the transcriptional regulation of miRNAs is identifying the primary transcript and transcriptional start site. While extensive information about the genomic position of miRNAs and their pre-miRNA hairpins is available, the long primary transcripts which give rise to intergenic miRNAs such as miR-31 are largely unknown. Processing of miRNA transcripts occurs co-transcriptionally, and the half-life of a mature primary transcript may be very short [164]. This appears to be the case for miR-31, as

no expressed sequence tag covers the miR-31 hairpin in mice. Sun *et al.* have published data indicating that miR-31 is expressed from a novel primary transcript in a mouse mesenchymal cell line [165]. However, we have not been able to confirm expression of this primary miR-31 transcript in primary CD8 T cells, nor have we been able to identify an alternative primary transcript by 5' rapid amplification of cDNA ends (RACE).

Without knowledge of the primary transcript, it is difficult to conclusively define proximal promoter elements for miR-31 in CD8 T cells. However, analysis of the miRNA transcriptional units in humans suggests that many intergenic miRNA promoters are within 10 kb of the miRNA hairpin [151]. Given this data, and the fact that the miR-31 hairpin is more than 25 kb from any known gene, we hypothesized that transcription factor binding sites within the vicinity of the miR-31 hairpin may play a role in regulating its expression. We computationally identified potential transcription factor binding sites in the surrounding sequence using the Lever algorithm, which searches non-coding regions for clustered, evolutionally conserved regulatory motifs [151]. This search found a predicted NFAT site within a conserved region upstream of the miR-31 hairpin, at base pairs 88559195-88559213 of mouse chromosome 4 (Figure 4.3A). To show that this predicted site was active in CD8 T cells, we performed chromatin immunoprecipitation (CHIP) with  $\alpha$ -NFAT antibodies before and after T cell activation, followed by detection of the predicted site by RT-PCR. We found a strong enrichment in NFAT binding to the predicted site after stimulation of CD8 T cells with  $\alpha$ -CD3/CD28 antibodies (Figure 4.3B). This data suggested that NFAT signaling could play a role in the transcriptional activation of miR-31 during T cell activation.

NFAT transcription factors are primarily regulated by calcium signaling through calmodulin and calcineurin. To connect miR-31 expression to NFAT regulation, we tested the effects of calcium signaling induction with the calcium ionophore ionomycin and suppression of calcium signaling through calmodulin with cyclosporine A (CyA). We found that treatment of



**Figure 4.3: Calcium signaling regulates miR-31 expression in CD8 T cells, potentially through an upstream NFAT binding site.** **A)** An NFAT binding site is located in a strongly conserved region upstream of the miR-31 hairpin. **B)** CHIP for NFAT binding to predicted site before and after stimulation of CD8 T cells with α-CD3/CD28. **C)** 24 hour treatment of CD8 T cells with α-CD3/CD28 or ionomycin is sufficient to induce miR-31. **D)** Blocking calcium signaling with cyclosporin A (CyA) strongly inhibits miR-31 expression induced by 24 hour treatment with α-CD3 or α-CD3/28.

CD8 T cells with ionomycin was sufficient to induce miR-31 (Figure 4.3C). Blocking calcium/calcineurin signaling with CyA during T cell activation with  $\alpha$ -CD3 strongly inhibited miR-31 expression (Figure 4.3D). These data indicate that calcium signaling is both necessary and sufficient for induction of miR-31 during early T cell activation.

### ***miR-31 target gene identification***

Identification of the mRNA targets of a miRNA is an essential step to understanding its function. miRNAs are capable of repressing hundreds of mRNAs, but accurate target identification and validation is difficult due to the partial complementarity seen in mammalian miRNA/mRNA pairs, the relatively small change in mRNA/protein levels of each target, and the poor understanding of the factors which regulate miRNA binding. Although most computational algorithms predict hundreds to thousands of targets for each miRNA, only a small fraction of these predictions represent valid targets, and no current target prediction method successfully predicts all experimentally validated miRNA/mRNA pairs [166]. Given the high false positive rate present in computational predictions, experimental validation is essential for identifying true targets.

MiRNA regulation reduces mRNA levels for the majority of target genes [167, 168]. Thus, ectopic expression of miRNAs followed by gene expression profiling is a useful technique for experimental target identification. To ectopically express miR-31 in primary CD8 T cells, we constructed a Plko.3g lentiviral vector expressing an experimentally validated Drosha substrate containing the miR-31 hairpin together with flanking sequence [169]. Empty Plko.3g vector was used as a control. Naïve, resting mouse T cells are very difficult to infect with lentiviral vectors. To enable lentiviral infection without inducing endogenous miR-31, we pre-cultured OT-I TCR transgenic CD8 T cells with IL-7 and IL-15 for 2 days prior to infection. Following infection with empty or miR-31 vector, we cultured cells for an additional 3 days in IL-2 before isolation of

GFP+ transduced cells by FACS. Gene expression in Empty- and miR-31-transduced T cells was profiled using Affymetrix 430 3' microarrays, and genes differentially expressed in miR-31 transduced samples were identified by Significance Analysis of Microarrays[154].

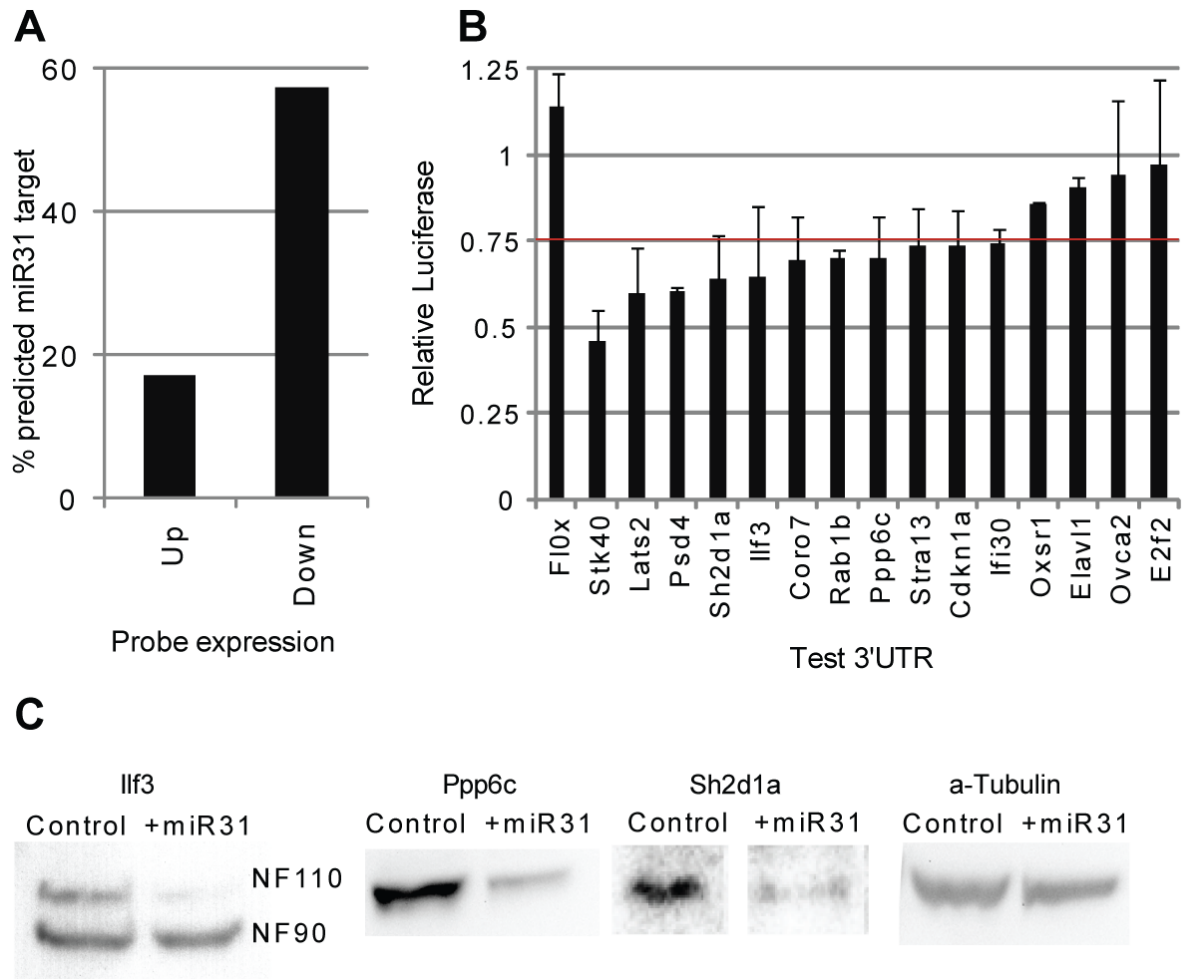
Given the inaccuracies of current target prediction methods, we utilized multiple computational algorithms to identify potential miR-31 target genes, including those that: require a stringent seed match (TargetsScan); allow flexible seed match with G/U wobble base pair binding (Miranda, PicTar); and allow seed match-independent motif identification (RNA22) [155-157, 159, 170]. Deep sequencing of Ago bound mRNA fragments has been proposed as a high-throughput technique for experimental target validation, and the Starbase prediction method is based on results from this technique [158]. Using the union of predictions from these 5 methods, we found that probes downregulated in the presence of miR-31 were strongly enriched in predicted miR-31 targets, with 57% (39/68) of downregulated probes representing predicted miR-31 targets (Figure 4.4A).

To determine if predicted targets downregulated in array data were directly regulated by miR-31, we cloned the 3'UTRs of 13 candidate target genes downstream of a luciferase reporter, and tested luciferase expression in the presence and absence of miR-31 (Figure 4.4B). We confirmed 11 3'UTRs in luciferase assays as being downregulated by more than 25% by miR-31 in at least two separate assays.. Additionally, three luciferase-validated miR-31 targets (Ilf-3, Ppp6c, and Sh2d1a) were confirmed to be endogenous miR-31 targets by Western blotting (Figure 4.4C). Three of our luciferase-validated target genes (Ppp6c, Lats2, and Stk40) have been previously published as miR-31 targets in human solid tumors [171-173]. Eight additional luciferase-validated genes (Psd4, Sh2d1a, Ilf3, Coro7, Rab1b, Ppp6c, Stra13, and Cdkn1a) represent novel miR-31 targets. These new targets significantly expand the number of validated mouse miR-31 targets and provide insight into the potential role of miR-31 in CD8 T cell activation.



**Figure 4.4: mir-31 target determination.** **A)** Affymetrix profiling of Empty (control) or miR-31 lentiviral vector-transduced CD8 T cells reveals a significant enrichment for predicted miR-31 targets among probes downregulated upon miR-31 ectopic expression. **B)** Protein expression from control (Fl0x) and test luciferase reporters containing the 3'UTRs of target genes identified by Affymetrix profiling in the presence or absence of miR-31 reveals strong downregulation of a subset of hits. Data presented are results from two independent assays. 3'UTRs were scored as downregulated if they were significantly different from control Fl0x in ( $p < 0.05$ ) each assay, and displayed an average downregulation of at least 25%. **C)** Western blots for miR-31-mediated repression of endogenous protein levels. Western blots for protein expression in T cell hybridoma lines transduced with empty or miR-31 lentiviral constructs. A-tubulin is presented as a loading control.

**Figure 4.4 (Continued)**



### ***miR-31 target gene functions***

One miR-31 target gene reveals an interesting aspect of miRNA-mediated regulation. Ilf3 (interleukin enhancer binding factor 3) encodes at least 4 splice variants, grouped into short NF90 and long NF110 gene products. The NF90 and NF110 mRNAs have different 3'UTRs, with only the NF110 variants containing a miR-31 site. In the presence of miR-31, expression of NF110 is selectively repressed leaving the expression of NF90 unaltered (Figure 4.4C). Ilf3 belongs to a cluster of transcription factors which bind to the proximal Il-2 promoter, and is required for the transcription and post-transcriptional stability of Il-2 and Il-13 [174, 175]. The NF90 splice form is thought to play the central role in cytokine transcription and stabilization. NF110 function is largely unknown despite experimental evidence indicating that NF110 is the most potent transcriptional activator of the Ilf3 family [176].

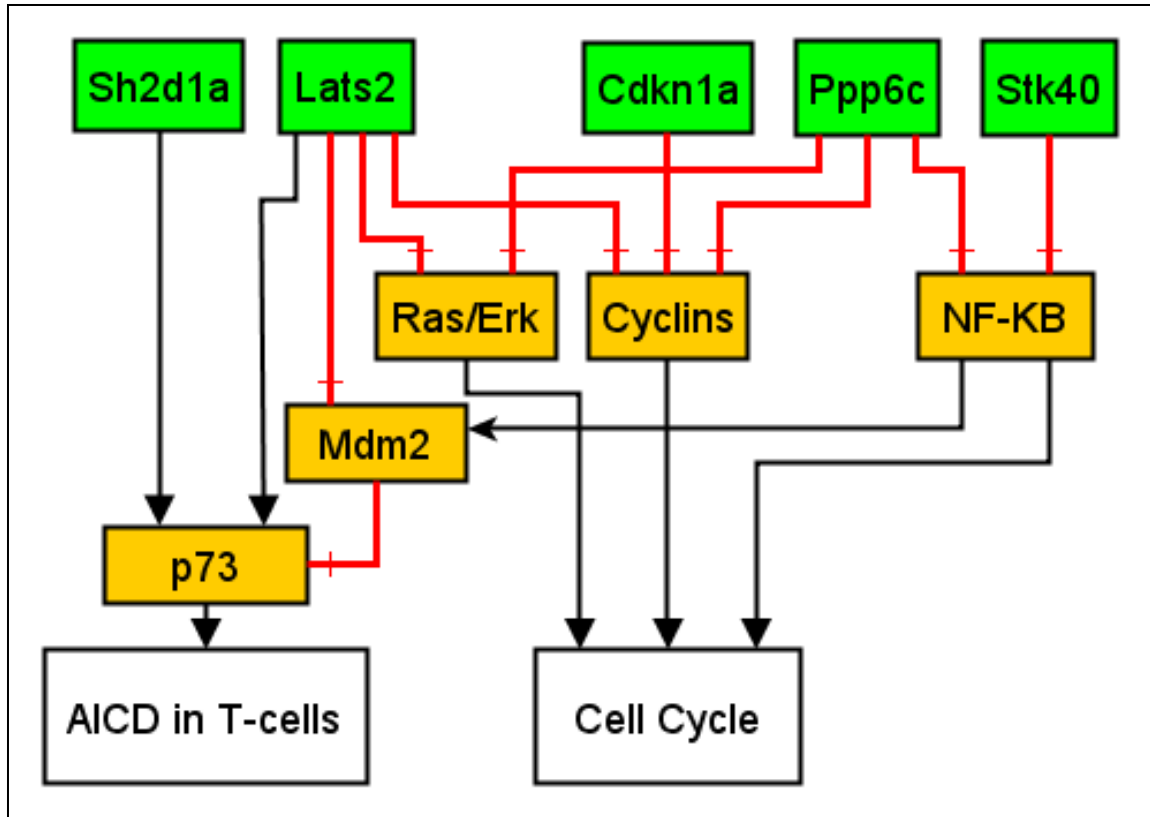
Of the confirmed target genes, the best studied are Ppp6c, Lats2, Sh2d1a, and Cdkn1a. Ppp6c is the catalytic subunit of protein phosphatase 6 (PP6). PP6 has been shown to regulate cyclin D1, stabilize I $\kappa$ B $\epsilon$ , and bind to the intracellular domains of CTLA4 and CD28 [177-179]. PP6 is also a major negative regulator of Tak1, the most commonly expressed Map3K7 member in the immune system [180]. Tak1 is essential for T cell development and homeostasis, where it regulates NF-K $\beta$  signaling and JNK/ERK activation [181, 182]. Lats2, large tumor suppressor homolog 2, is a Ser/Thr kinase which plays an important role in the Hippo signaling pathway [183]. Expression of Lats2 is linked to p53/p73 stabilization through negative regulation of the MDM2 E3 ubiquitin ligase, cell cycle arrest, and transcriptional upregulation of negative regulators of the MAPK pathway [184, 185]. Sh2d1a is a Sh2 domain containing adapter protein which is exclusively expressed in lymphocytes, with demonstrated expression in T cells, NK cells, and some B cell populations. Sh2d1a mediates signals from the signaling lymphocytic activation molecule family of immunomodulatory receptors. Knockout of Sh2d1a in the mouse leads to hyperproliferative T cell responses, defective activation induced cell death (AICD), and

impaired T cell-B cell interactions [186, 187]. The cyclin dependent kinase inhibitor Cdkn1a (also known as p21, Waf1, or Cip1) is an negative regulator of cyclin activity and proliferation with important functions in the control of memory T cell homeostasis and secondary expansion [188]. Less is known about Stk40. However, it was originally identified as an inhibitor of TNF-triggered NF- $\kappa$ B activation and p53 activity [189]. miR-31 regulation leads to a small effect on the expression of each individual gene. However, if multiple target genes affect a common pathway, then miR-31 induction could significantly contribute to the phenotypic changes observed during T cell activation.

These miR-31 regulated genes are involved in intersecting pathways with important functional roles in T cell activation. A model for the interaction between miR-31 target genes and downstream pathways is presented in Figure 4.5. Multiple target genes are negative regulators of ERK/MAPK and NF- $\kappa$ B pathways, and we predict that these pathways will be de-repressed upon miR-31 induction. A second major focus is the p73 member of the p53 family of proteins, an important pro-apoptotic factor in T cells. Lats2 and Sh2d1a have both been associated with p73 induction or stabilization, and NF- $\kappa$ B signaling is crucial for preventing p73-driven cell death during T cell activation [186, 190, 191]. miR-31 induction thus may act to protect T cell from p73-mediated apoptosis through direct repression of Lats2/Sh2d1a coupled with indirect activation of NF- $\kappa$ B.

### ***miR-31 Activates MAPK/ERK and Induces CD69 expression in a Murine T-hybridoma***

Our gene pathway model predicts that miR-31 de-represses ERK/MAPK signaling. We transfected a murine T-hybridoma cell line with empty and miR-31-expressing lentiviral vectors in order to test the effects of ectopically expressing miR-31. Surprisingly, expression of miR-31 alone was sufficient to induce significant increases in MAPK pathway phosphorylation. The most



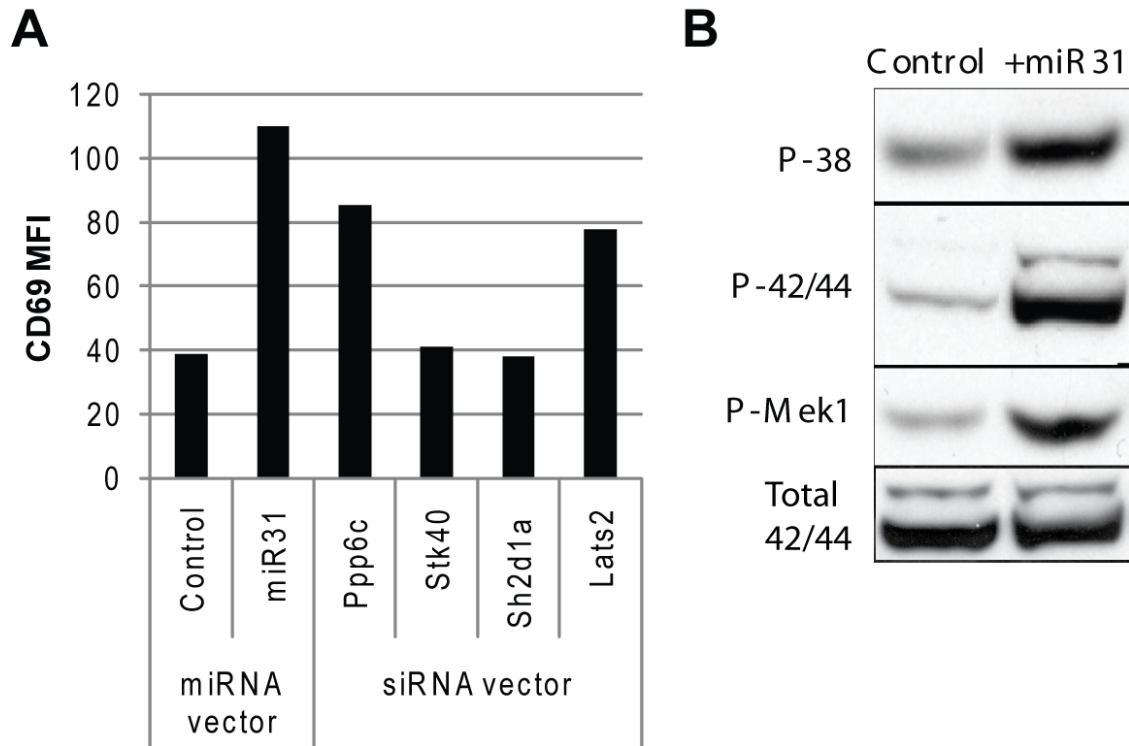
**Figure 4.5: Pathways regulated by confirmed miR-31 targets.** miR-31 target genes are in green, affected genes and signaling pathways are in orange. Inhibitory pathways are depicted in red, activating pathways are depicted in black.

dramatic increases were seen in p42/44 ERK1/2 phosphorylation, but significant increases were also seen in MEK1 and p38 phosphorylation (Figure 4.6B). The expression of the CD69 activation marker is driven by ERK/MAPK induction of AP1 transcription factor activity [192], and ectopic expression of miR-31 was sufficient to induce CD69. Lentiviral siRNA-mediated knockdown of two miR-31 targets, Lats2 and Ppp6c, phenocopied the effect of ectopic miR-31 expression on CD69 (Figure 4.6A). This data supports a model where miR-31 expression de-represses ERK/MAPK activation, and indicates that repression of Lats2 and Ppp6c by miR-31 may be the major mediator of this effect.

### ***miR-31 inhibition in primary mouse T cells***

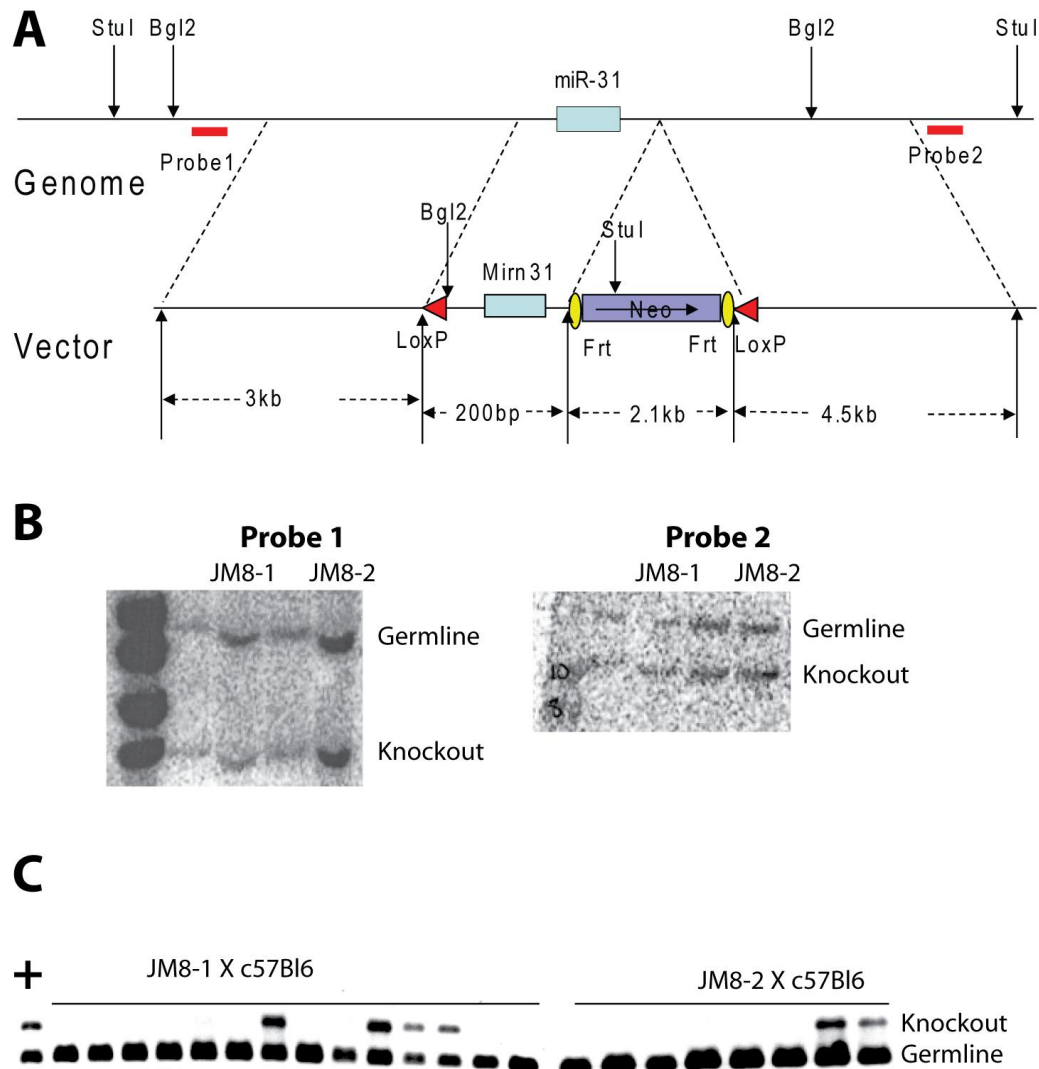
High levels of miR-31 expression are seen in CD8 T cells early after activation, and persist in memory populations. To experimentally investigate the physiological importance of miR-31 induction during the immune response, it is essential to inhibit miR-31 activity. The primary experimental approach for inhibiting miRNA function is the transfection of single-stranded antisense inhibitory oligonucleotides, which bind stoichiometrically to mature miRNAs and block their function. However, the low transfectability of T cells presents a significant barrier to miR-31 inhibition using this method. A number of publications have proposed experimental approaches to deliver inhibitory oligonucleotides to T cells [193-196]. In our hands, none of these methods effectively blocked miR-31 function in CD8 T cells. Genomic deletion is the definitive means for inhibiting gene expression, and we are in the process of developing miR-31 knockout mice.

miR-31 was first cloned from embryonic stem cells and is expressed in solid tissues, raising the potential that complete loss of miR-31 could lead to developmental defects or an embryonic lethal phenotype [197]. To control for this possibility, we utilized a conditional knockout strategy, which places LoxP sites in the non-conserved regions flanking the miR-31 hairpin (Figure 4.7A). A first round of c57bl/6 ES cell derived chimeras did not lead to germline



**Figure 4.6: miR-31 expression in a T-hybridoma cell line leads to induction of CD69**

**expression and ERK/MAPK signaling. A)** Expression of miR-31 or knockdown of miR-31 targets Ppp6c and Lats2 induces CD69 expression in cultured murine T-hybridoma cells. Cells were stained with  $\alpha$ -CD69, and gated on GFP<sup>+</sup> cells. **B)** Western blot for phospho-MAPK pathway members p38, p42/44 Erk1/2, Mek1, and control total p42/44 Erk1/2 in T-hybridoma cells transduced with control plko.3G or miR-31 expressing plko.3G.



**Figure 4.7: Conditional knockout of miR-31.** **A)** miR-31 targeting construct inserts LoxP sites flanking miR-31 hairpin. Two locations for Southern blot probes outside of flanking sequence and relevant restriction enzyme digestion sites are shown. **B)** Southern blots of ES cell DNA digested with BglII for probe 1 and StuI for probe 2 confirm insertion of targeting construct in multiple JM8 ES cell line clones, including JM8-1 and JM8-2. **C)** PCR screening over the proximal LoxP site confirms germline transmission of the conditional miR-31 knockout allele in multiple offspring of chimeras derived from JM8-1 and JM8-2 ES cell lines.



transmission, but by utilizing the recently developed c57bl/6 JM8 ES cell line [198], we were able to efficiently generate recombinant ES clones (Figure 4.7B). Chimeras derived from 2 independent JM8-derived clones displayed germline transmission (Figure 4.7C). We are currently breeding these mice to remove the neomycin resistance cassette, followed by crosses with Lck-cre to induce a T cell-specific knockout and with Zp3-cre to induce a complete knockout. These knockouts will allow us to unequivocally determine the role of miR-31 in immune function.

## Discussion:

In this study we present a novel miRNA expression signature in early stages of CD8 T cell activation. Our data reveal that CD8 T cells upregulate and downregulate a large number of miRNAs early in activation. These data stand in contrast to two previous studies of miRNA expression during *in vitro* differentiation of CD8 T cells into effector memory and central memory cells, which reported relatively modest changes in miRNA expression [142, 143]. Our results are consistent with observations in CD4 T cells where large changes in miRNA expression early after activation, followed by relatively small differences between memory subsets, were observed [37, 49]. There is considerable overlap between the individual miRNAs which are reported to be altered in early CD4 T cell activation and our CD8 T cell activation profile, including downregulation of miR-181a, miR-150, and the miR-26, miR-29 and miR-30 families, as well as upregulation of the miR-17-92 cluster, miR-146, and miR-155. Our data shows transient alterations in the expression level of many miRNAs, supporting a significant role for miRNA-mediated regulation in early stages of T cell activation.

We observe strong upregulation of miR-31 in CD8 T cells early after activation, with sustained expression in memory T cells. This miR-31 induction is calcium responsive and subject to inhibition by cyclosporin A. We observed recruitment of NFAT transcription factors to a conserved binding site upstream of the miR-31 hairpin upon T cell activation, suggesting that NFAT regulates miR-31 expression. However, NFAT transcription factors typically require a second transcription factor for optimal DNA binding and transcriptional co-activation. AP1 is the classic NFAT partner, but others have been described, including members of the MAF and GATA transcription factor families [199]. Additional research is necessary to define the miR-31 promoter in mice, and closely examine the role of NFAT family members and transcriptional partners in regulating pri-miR-31 transcription.

Other groups have observed differential miR-31 expression in T cell subsets. Wu et al. reported miR-31 expression in *in vitro* differentiated CD8 central memory cells. In contrast to our results, which indicated that *in vitro* differentiated effector memory T cells maintained high levels of miR-31 expression, Wu et al. failed to detect miR-31 in effector memory T cells, possibly due to the technical limitations of the cloning techniques utilized to profile miRNA expression [143]. Almanza et al. compared miRNA expression between *in vitro* differentiated effector memory and central memory T cells and observed higher miR-31 expression in effector memory T cells, consistent with our data [142]. miR-31 has also been identified as being upregulated in total lymphocytes isolated from Il-10 knockout mice, a model for Th1 type autoimmunity, and in lymphocytes from mouse models of lupus [200, 201]. It is likely that these results reflect elevated miR-31 levels in activated autoimmune T cells.

A function of miR-31 has been previously investigated in human T cells. Rouas et al. have demonstrated that human Treg cells express low levels of miR-31, and demonstrated direct repression of the crucial Treg transcription factor Foxp3 by miR-31 [202]. We observed elevated miR-31 levels in mouse Treg cells, and the miR-31 binding sites in the human FOXP3 3'UTR are poorly conserved in mice [156], suggesting that this role for miR-31 may be specific to human Treg cells. While little is known about the role of miR-31 in T cells, there have been a number of recent publications on the role of miR-31 in human solid tumors. In this context, miR-31 expression can function as either an oncogene or a tumor suppressor, depending on cell type. For example, miR-31 expression inhibits the proliferation of ovarian carcinomas, but functions as an oncogenic miRNA in lung cancer by inhibiting tumor suppressors, including LATS2, which we also identify as a target in mouse T cells [171, 172]. The contradictory outcomes of miR-31 expression illustrate an important aspect of miRNA function: the effects of a miRNA are entirely shaped by the mRNA targets expressed in a particular cell type. This is best demonstrated by studies on the role of miR-31 on tumor migration and metastasis. In breast cancer and

endometrial cancer-associated fibroblasts, miR-31 functions as an inhibitor of metastasis by repressing a group of genes, including the homeobox SATB2 and the small GTPase RhoA [203, 204]. However, in colon carcinoma, miR-31 promotes cell motility by repressing TIAM1, a guanine exchange factor of the Rac GTPase [205]. These data demonstrate the functional flexibility of gene regulation by miRNAs and emphasize the need to define the miRNA targets for each individual cell type.

We identified 11 genes as targets of miR-31-mediated repression in CD8 T cells. Among these targets are *Lats2*, *Ppp6c*, and *Cdkn1a*, which have known roles as tumor suppressors, supporting our model for miR-31 promotion of activation, proliferation, and survival after T cell activation [178, 190, 206]. In addition, published data indicate that *Cdkn1a* is also regulated by the activation-induced miR-17 family [207]. Repression of *Cdkn1a* by multiple miRNAs may play an important role in reversing the quiescent state of naïve T cells and priming cells for rapid division.

In conclusion, we show large alterations in miRNA expression in early stages of CD8 T cell activation. We determined the signaling pathways transcriptionally controlling the expression of one upregulated miRNA, miR-31, and examined its expression in detail in differentiated T cells and other immune cell types. We identified and validated miR-31 targets in primary CD8 T cells, including 8 previously unreported target genes. From published information on gene function, we propose a model whereby induction of miR-31 primes CD8 T cells for activation by repressing pro-apoptotic factors and negative regulators of cyclin, NF- $\kappa$ B, and MAPK signaling. Increased phosphorylation of Erk/MAPK pathway members in mouse T-hybridoma cells after ectopic miR-31 transduction supports our model for miR-31-mediated de-repression of T cell activation pathways. Our results indicate that miR-31 may play important roles in both early T cell activation and long term memory T cell subsets.

## **Chapter 5**

### **General discussion**

### ***miRNAs are multipurpose regulators***

One of the most striking aspects of miRNA research is the many phenotypes associated with a single miRNA or miRNA family. The potential for repression of many genes through short recognition sequences intersects with shifting mRNA transcriptomes to allow a single miRNA to play distinct functional roles in different tissue types and developmental stages. In this dissertation, we explored miRNA expression and function in three separate but related systems: murine hematopoietic development, murine CD8 T cell activation, and human normal B cells and CLL cells. While miRNA expression patterns restricted to each system are discussed in detail in the relevant chapter, several miRNAs are involved in multiple systems. The most notable of these are the miR-181 and miR-29c family, which we identified as differentially expressed during hematopoietic development, T cell activation, and in human hematopoietic malignancies.

### ***miR-181***

The four members of the miR-181 family in mouse and human are derived from 6 hairpins arranged in 3 separate genomic clusters: miR-181a-1 and miR-181b-1; miR-181a-2 and miR-181b-2; and miR-181c and miR-181d. miR-181 was one of the first miRNAs to be implicated in hematopoietic development. The first publication from Chen *et al.* indicated that miR-181a was highly expressed in mouse hematopoietic tissues, including bone marrow and thymus. Ectopic expression of miR-181a in mouse HSC promoted B cell development and impaired CD8 T cell development [36]. These results were confusing, given the strong expression of miR-181a observed in the thymus by our group and other researchers. Later research has shown that, under the proper developmental conditions miR-181a enhances both T cell and NK lineage differentiation [87, 208]. We have demonstrated that commitment to lymphoid lineage development correlates with expression of miR-181 family members during murine hematopoiesis during both T and B lineage development (Figure 2.1). Given the existing data, the

best model for miR-181 function during lineage determination is that it acts to promote general lymphoid development, with the specific lymphoid lineage determined by other environmental cues. When ectopically expressed in HSC in the bone marrow, an environment suitable for B lineage but not T lineage development, miR-181 increases commitment to the B cell lineage. When expressed in conditions permissive for T cell or NK cell development, miR-181 assists in driving development of these lineages. Little is known regarding the miR-181 target genes involved in driving early lymphoid commitment, but a recent report implicated miR-181a repression of nemo-like kinase, an inhibitor of Notch signaling, in promotion of NK cell differentiation [208]. As Notch signaling is also an essential step in T lineage commitment and development, silencing of nemo-like kinase by ectopically expressed miR-181a could also lead to the increased T lineage development observed *in vitro* [87].

In contrast, the role of miR-181 in modulating late T cell development has been well studied. Although miR-181 family expression in DP cells is significantly lower than in DN stages of thymocyte development (Figure 2.2B and [79]), it plays an important role in modulating positive and negative selection. An elegant paper by Li *et al.* demonstrated that miR-181a repressed a group of inhibitory phosphatases, increasing T cell receptor sensitivity and helping to set the threshold for positive and negative selection [79]. Notably, when we examined miR-181 family expression during SP thymocyte maturation using CD24 expression to separate early SP cells, which are still undergoing negative selection, from late SP cells preparing to exit the thymus, we observed high levels of miR-181a expression in early SP cells. Late SP cells expressed lower miR-181a levels, similar to those observed in mature T cells (Figure 2.2B). This expression pattern correlates with continued sensitivity to negative selection in early SP cells. Some miR-181 family members are also expressed in mature murine T cells and B cells (Figure 2.1 and 4.1). Li *et al.* identified an important role for miR-181a feedback in regulation of the T cell response, with stimulation through the TCR downregulating miR-181 expression, leading to

lower TCR sensitivity [79]. This data is consistent with our results in mouse T cells and human B cells, where we observed miR-181a downregulation in activated mouse T cells and miR-181a and miR-181b downregulation in activated human B cells.

In human malignancies, miR-181 family expression is high in human malignancies derived from lymphoid progenitors, and in some myeloid progenitor tumors, but very low in CLL (Figures 2.4 and 3.1). This data partially recapitulates developmental-stage specific expression that we observe in the mouse. The low expression of miR-181 in CLL is particularly interesting, given some intriguing aspects of CLL biology. CLL cells display preferential IgV<sub>H</sub> usage and often express closely homologous CDR3 sequences on both heavy and light BCR chains [209, 210]. These observations have raised the possibility that stimulation by an auto-antigen may play a role in CLL disease. This theory is compatible with our observed miRNA profile, which links CLL cells to activated B cells (Figure 3.2). It also offers a potential explanation for the hypo-responsive BCR response observed in CLL [99]. Chronic antigen signaling in human disease such as leprosy has been associated with induction of anergic T cells, an effect which is partially mediated by a loss of miR-181a expression [211]. Extending this model to CLL, chronic BCR signaling in CLL could lead to the upregulation of miR-155 and downregulation of miR-181a observed in CLL, with the loss of miR-181a feedback inducing the hypo-responsive CLL BCR response.

### ***miR-29***

The miR-29 miRNA family is present in two genomic *loci* in mice and humans, one of which contains miR-29b-1 and miR-29a, and a second containing miR-29b-2 and miR-29c. Our data and other studies indicate that miR-29 family expression is specifically induced and repressed at specific stages of hematopoietic ontogeny. Human and mouse HSC express high levels of miR-29a, and miR-29a expression promotes cellular self-renewal [212, 213]. During



mouse T cell development we observe low expression of miR-29a and c in thymocytes, and significantly higher levels in mature T cells and B cells (Figure 2.1C). Thus, miR-29 family expression is lost at some point between HSC and T lineage development in the thymus, and then re-induced during T cell maturation. This dynamic expression pattern continues during T and B cell activation. CD8 T cell activation leads to downregulation of miR-29a and c (Figure 4.1), and this downregulation is linked to enhanced interferon- $\gamma$  production [148, 149]. Transcription of miR-29b-1 and miR-29a has been shown to be repressed by c-Myc, hedgehog, and NF- $\kappa$ B signaling, consistent with the observed downregulation after T cell activation [214]. Expression of miR-29 family members in human B cells was more complex, with miR-29 b and c downregulated in response to  $\alpha$ -BCR or CD40L stimulation, and upregulated in response to TLR stimulation. miR-29a displayed the opposite pattern, with upregulation in response to  $\alpha$ -BCR or CD40L, and downregulation in response to TLR ligands (Table 3.1). Profiling of miRNA expression in human memory B cell subsets found higher expression of miR-29a, b, and c in memory B cells compared to centroblasts [215]. The role of miR-29 family members in B cell activation and memory formation is currently unknown.

The role of miR-29 in cancer is complex and still not fully understood. We observed higher expression of all miR-29 family members in CLL vs. normal B cells (Figure 3.1), implying that miR-29 expression might play a role in CLL oncogenesis. In support of this possibility, a mouse model with conditional overexpression of miR-29a and miR-29b-1 in B cells lead to development of a CLL-like disease, and ectopic expression of miR-29a in mouse HSC was sufficient to induce AML [212, 216]. However, in CLL, our data associated low miR-29c expression with poor clinical markers (ZAP70<sup>+</sup> and IgV<sub>H</sub> unmutated) and shorter time to first therapy (Figure 3.4 and Table 3.2). Other published profiles comparing aggressive to indolent CLL have also linked low expression of miR-29a, b, and c to an aggressive disease course [217]. A potential tumor suppressor role for miR-29 is supported by data indicating miR-29b represses

Tcl1, a critical oncogene in aggressive CLL [218]. In addition, low miR-29 family expression has been described in multiple other types of cancer, including lung cancer, cholangiocarcinoma, and nasopharyngeal carcinomas [114, 219, 220]. Given that the miR-29 family is expressed dynamically during hematologic development, it is possible that miR-29 expression in CLL is a marker for differentiation stage. The observed association between lower miR-29 and aggressive tumors could be due to a connection between low miR-29 expression and less differentiated tumors, a hypothesis supported by the association between low miR-29c and IgV<sub>H</sub> unmutated CLL. Future research is necessary to separate correlations between miR-29 and aberrant differentiation from the roles of the miR-29 family in oncogenesis or tumor suppression.

### ***Future research into the role of miRNAs in hematopoietic ontogeny***

Future research on miRNA function during hematopoietic development, the immune response, and hematopoietic malignancies will be aided by identification of the signaling pathways and transcription factors which regulate pri-miRNA expression. After expression, the second important aspect is miRNA target identification and validation. New techniques offer the promise of significant improvements in high throughput target identification. The final challenge lies in connecting miRNA targets to miRNA dependent phenotypes.

### ***Determination of miRNA precursors:***

Identification of intergenic pre-miRNA sequences remains a problem for analyzing transcriptional control of miRNAs, as we observed for miR-31. We were not able to identify a primary transcript for miR-31 through directed approaches, and no potential primary transcript has been deposited into GenBank by another group despite miR-31 expression in well studied tissues such as mouse kidney and ES cells. Recent technical advances in deep sequencing hold promise for identifying miRNAs expressed from the introns of currently unknown transcripts. As knowledge of the hematopoietic transcriptome increases, these cryptic transcripts should be

revealed. Identification of primary transcripts containing an exonic miRNA could be more difficult, given the potential for an exceedingly short half-life for these pri-miRNAs. Computational analysis of histone methylation patterns is a promising approach to identifying active promoter regions proximal to the miR-31 hairpin [221]. A potential experimental approach for isolating exon-encoded pre-miRNA transcripts is conditional knockout of the miRNA biogenesis enzyme Drosha. Without Drosha cleavage, these transcripts should be stabilized and available for cloning and sequencing. Once the primary transcript has been identified, the same techniques developed to analyze protein coding genes can be used to identify miRNA transcriptional regulators.

### ***Isolation of miRNA targets***

It is important to determine miRNA targets in the cell type of interest. At the most basic level, the interplay between target and miRNA expression determines miRNA function, as has been clearly shown for miR-31 dependent phenotypes in human solid tumors. In addition to gene expression, the presence of miRNA target sites can be altered by splicing dependent 3' exon switching and splicing independent use of alternative polyadenylation sites. We observed differential miR-31 repression of splice variants from the *Ilf3* gene, and detailed microarray profile analysis by Sandberg *et al.* found that both exon switching and use of alternative polyadenylation sites is a common feature of T cell activation [144]. These results emphasize the need to identify miRNA targets as close to the cell type of interest as possible. Within the hematopoietic system, the limited biomass and limited transfectability of most cell types poses technical challenges to miRNA target identification.

Techniques for high-throughput miRNA target identification are typically based on miRNA overexpression or knockdown/knockout followed by mRNA expression profiling through microarray or deep sequence analysis, biochemical isolation of RISC/miRNA associated

mRNA, or proteomic analysis of total protein levels. Of these, mRNA expression profiling remains the most commonly used method. In our hands, profiling was an effective means of identifying novel miRNA targets. Our data comparing miR-31 to control vector transduced CD8 T cells found that 57% of downregulated probes were predicted miR-31 targets. This data is consistent with ectopic expression of other miRNAs. For example, after ectopic expression of miR-124 in Hela cells, 65% of downregulated mRNAs contained a 7-mer match to the seed region of miR-124 [222]. However, this effect is not observed in all systems. Boldin *et al.* did not observe significant upregulation of predicted miR-146a targets in miR-146a knockout macrophages, even for targets shown to be regulated at the protein level [50]. The relatively modest target downregulation induced by miRNA binding makes detection difficult, and some targets may be regulated exclusively at the level of translation. Thus, expression profiling may not be sufficient to identify all targets.

Alternative techniques for detection of miRNA targets are in development. Stable isotope labeling of cultured cells followed by detection by mass spectrometry can detect downregulation of miRNA targets at the protein level [167, 168]. This technique has provided essential information regarding the correlation between changes in protein level and mRNA levels in miRNA repressed mRNAs, but the expense, technical difficulty, and high biomass required currently renders it infeasible for most miRNA studies. A second approach is to identify miRNA or RISC bound mRNAs. Co-purification of mRNAs bound to RISC has a proven record in identifying true miRNA targets [223, 224]. The primary problem with this technique is that it enriches for all RISC-associated mRNAs, making identification of the targets of a particular miRNA difficult. A recently developed extension of this technique known as Ago HITS-CLIP (high throughput sequencing by cross-linking and immune-precipitation) partially addresses this problem [225]. This method involves Ago precipitation followed by digestion of unprotected RNA, sequencing of Ago protected fragments, and analysis of predicted binding sites within the

protected sequence. This method is a dramatic improvement in specificity, although Ago protected fragments are 40-60 nt long, and can contain multiple miRNA binding sites. While promising, attempts to identify targets of a specific miRNA by directly precipitating mRNAs bound to a labeled miRNA have met with limited success [226, 227]. These improvements in miRNA target algorithms both aid in identifying miRNA targets, and also hold the potential to further elucidate the currently unknown factors which separate functional miRNA target sites from similar non-target sequences.

### ***Correlating miRNA expression, miRNA targets, and miRNA dependent phenotypes***

The most common technique for connecting miRNA targets to a miRNA dependent phenotype involves repressing the level of individual targets with siRNAs, or ectopically expressing miRNA-insensitive target variants. For example, we were able to partially phenocopy the pro-activation effect of miR-31 in T-hybridoma cells by silencing the Lats2 or Ppp6c target genes (Figure 4.6), and the increase in NK cell development due to miR-181a can be phenocopied by silencing of nemo-like kinase [208]. For miRNAs with a known phenotype which is amenable to high throughput screening, siRNA screens can be used to isolate phenotype-associated genes, offering a subset of potential miRNA targets which are already linked to a phenotype. A recent paper on the role of miR-19 in T-ALL identified 4 novel miR-19 target genes among the set of 8 target genes where siRNA silencing phenocopied miR-19 expression [228]. However, many miRNA dependent phenotypes depend on concurrent low level repression of many individual genes, and cannot be duplicated by altering the expression of a single target. Silencing of individual target genes was not able to recapitulate the effect of miR-181a on TCR sensitivity [79], and phenocopy of the anti-metastatic effect of miR-31 in breast cancer required silencing of 3 target genes [229]. Where the roles of target genes are well understood, computational analysis of gene networks can aid in isolating critical target genes [230]. Experimental identification of

genes responsible for multi-gene phenotypes could be accomplished by screening with Pol-II based multi-siRNA libraries containing random combinations of predicted targets, or through the expression of multiple target protectors, which compete for miRNA binding to individual target genes [231, 232]. Despite technical advances, tracing the connection between miRNAs, their target genes, and miRNA dependent phenotypes will remain the primary challenge in miRNA research.

## ***Conclusions***

In this dissertation, we present data on miRNA expression in three systems: murine hematopoietic development, murine CD8 T cell activation, and human normal B cells and CLL cells. In each system, we have investigated a subset of miRNAs in detail. In murine hematopoiesis, we observed that 2 microRNA families, miR-181 and miR-128 were expressed in thymocytes. We demonstrated that these miRNA families are induced during commitment to lymphoid lineage and that expression is maintained throughout T cell development in the thymus and B cell development in the bone marrow. In human B cells and CLL, we identified common miRNA expression signatures in CLL and B cell activation, as well as miRNAs which correlate with CLL clinical markers and disease progression. In murine CD8 T cells, we identified a novel profile for miRNA expression during CD8 T cell activation, and investigated the function of one little-known activation induced miRNA, miR-31 in detail. We determined miR-31 expression patterns in CD4 and CD8 T cell subsets, and show that miR-31 induction during T cell activation is regulated by signaling through calcium and calcineurin. We identified miR-31 targets in primary CD8 T cells, a subset of which were previously identified in human solid tumors. Based on these targets, we hypothesize that miR-31 expression promotes T cell survival, activation, and proliferation.

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